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Further Observations on the Effect of Anoxia on Histamine Release from Guinea-Pig and Rat Lung Tissue in Vitro

by

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Abstract

DIAMANT B *Further observations on the effect of anoxia on histamine release from guinea pig and rat lung tissue in vitro* Acta physiol scand 1962 *56* 1—16 — Histamine release was induced *in vitro* by antigen from sensitized guinea pig and rat lung tissue and by compound 48/80 and *Ascaris* extract from non sensitized rat lung tissue Pre exposure of the lung tissue to nitrogen anoxia in the absence of glucose (pre incubation) markedly inhibited histamine release subsequently elicited under nitrogen in the presence of glucose, or under oxygen in its absence whereas the same procedure had a comparatively smaller effect on histamine release induced under oxygen in the presence of glucose Washing the lung tissue after pre incubation enhanced the histamine release subsequently induced under nitrogen with glucose whereas histamine release under oxygen with or without glucose was less affected by the washing procedure Rat and guinea pig lung tissue exposed to compound 48/80 and antigen respectively during pre incubation was found to be insensitive to a second exposure to the same histamine releaser The results are explained on the assumption that high energy compounds available at the time of exposure of the lung tissue to the histamine releasing agents are of consequence for histamine release to proceed They also suggest that the initial reaction (coupling of antigen to antibodies and of compound 48/80 to active sites) occurs without involvement of high-energy compounds

It has repeatedly been shown that glucose counteracts the inhibiting effect of anoxia on the release of histamine induced *in vitro* by extracts of *Ascaris suis* (DIAMANT 1960, 1961 a) and by compound 48/80 (DIAMANT and UVNÄS 1961) from rat lung, and by antigen from sensitized rat and guinea pig lung (DIAMANT 1961 b). A similar effect of glucose has been reported on histamine release caused by compound 48/80 from cat skin (WESTERHOLM 1960) and from rat diaphragm (ROTHSCHILD, VUGMAN and ROCHA E SILVA 1961).

These findings were suggested to reflect metabolic changes in the mast cells, since it was observed that degranulation of mast cells in rat mesentery pieces induced by compound 48/80 was blocked by anoxia, as well as by metabolic inhibitors, an effect that was prevented by the presence of glucose (DIAMANT and UVNÄS 1961, ROTHSCHILD, VUGMAN and ROCHA E SILVA 1961). This effect of glucose has been confirmed under anoxic conditions when mast cell degranulation was induced by antigen, chymotrypsin and by phosphatidase A (UVNÄS 1961 a).

The present investigation deals mainly with the action of oxygen, nitrogen and glucose on the *in vitro* release of histamine from lung tissue pre-exposed to nitrogen in a glucose-free medium ("pre-incubation").

Table I. Procedures used in the present investigation for incubating the lung tissue. + between 2 time intervals denotes continued incubation. ‖ between 2 time intervals denotes washing between "pre-incubation" period and reincubation period. Nitrogen (N_2), oxygen (O_2), glucose and histamine releaser (hist. rel.) introduced at the beginning of corresponding time interval.

I	15 min glucose O_2 + 20 min hist. rel. O_2
II	15 min O_2 + 20 min hist. rel. O_2
III	15 min glucose N_2 + 20 min hist. rel. N_2
IV	15 min N_2 + 20 min hist. rel. N_2
V	0, 7.5, 15, 22.5 or 30 min N_2 + 15 min glucose O_2 + 20 min hist. rel. O_2
VI	0, 7.5, 15, 22.5 or 30 min N_2 + 15 min O_2 + 20 min hist. rel. O_2
VII	0, 7.5, 15, 22.5 or 30 min N_2 + 15 min glucose N_2 + 20 min hist. rel. N_2
VIII	35 min N_2 ‖ 15 min glucose O_2 + 20 min hist. rel. O_2
IX	35 min N_2 ‖ 15 min O_2 + 20 min hist. rel. O_2
X	35 min N_2 ‖ 15 min glucose N_2 + 20 min hist. rel. N_2
XI	35 min N_2 ‖ 15 min glucose O_2 + 20 min hist. rel. O_2
XII	35 min N_2 15 min O_2 + 20 min hist. rel. O_2
XIII	35 min N_2 15 min glucose N_2 + 20 min hist. rel. N_2
XIV	15 min N_2 + 20 min hist. rel. N_2 ‖ 15 min glucose O_2 + 20 min hist. rel. O_2
XV	15 min N_2 + 20 min hist. rel. N_2 ‖ 15 min glucose N_2 + 20 min hist. rel. N_2
XVI	15 min N_2 + 20 min hist. rel. N_2 ‖ 35 min glucose O_2
XVII	35 min N_2 ‖ 35 min glucose O_2
XVIII	30 or 60 min O_2 + 15 min glucose N_2 + 20 min hist. rel. N_2
XIX	15, 30 or 60 min glucose N_2 + [illegible] hist. rel. N_2
XX	30 min N_2 + 15, 30 or 60 [illegible] N_2

Material and Methods

An account has been given earlier of the procedure for active sensitization of rats and guinea pigs (DIAMANT 1961 b) and for preparation of the lung tissue prior to incubation (DIAMANT and UVNÄS 1961). The lung tissue was incubated in phosphate medium (1 ml/100 mg tissue) with a composition identical to that previously described (DIAMANT and UVNÄS 1961). The initial pH of the phosphate medium ranged from 7.1 to 7.3 in the various experiments.

The details of the incubation procedures used in the different experiments have been given in Table I and the Roman figures appearing in the text refer to this table.

The final concentration of the histamine releasers in the incubation medium was as follows: Compound 48/80 35 μg/ml, antigen (crystallized egg albumin) 1 mg/ml, *Ascaris* extract 3 mg/ml. The glucose concentration was 5.6×10^{-3} M.

Incubation was performed in stoppered Erlenmeyer flasks at 37° C under continuous shaking and during exposure to oxygen or nitrogen in the presence or absence of glucose for 15 min prior to addition of the histamine releaser. After contact with the histamine releaser for 20 min under continuous exposure to the same gas as during the preceding 15 min, the incubation fluid was withdrawn (I, II, III and IV).

(1) The effect was investigated of pre-exposing rat lung tissue to nitrogen in the absence of glucose for 7½ to 30 min ("pre-incubation") on subsequently elicited histamine release. Following the various "pre-incubation" periods incubation was continued under oxygen in the presence (V) or absence (VI) of glucose, or under nitrogen in the presence of glucose (VII) prior to addition of the histamine releaser (*Ascaris* extract, compound 48/80 or antigen). In each experiment the histamine release found without "pre-incubation" was taken as 100 %, and the values obtained after "pre-incubation" were correlated to this value.

(2) The effect of washing rat and guinea pig lung tissue after 35 min "pre-incubation" on histamine release subsequently induced by compound 48/80 and antigen, respectively, under oxygen with (XI) or without (XII) glucose and under nitrogen with glucose (XIII) was also investigated. Thus, the lung tissue was incubated twice for 35 min. Between incubations it was washed at room temperature with 10 ml of glucose-free phosphate medium (|| in Table I). During the washing procedure the lung tissue was in contact with air. The histamine release from lung tissue treated in this way (XI, XII and XIII) was compared with the release obtained from lung tissue identically incubated except for the washing procedure (VIII, IX and X) as well as with the release from lung tissue incubated without "pre-incubation" (I, II and III).

(3) The effect of exposing rat and guinea pig lung tissue to compound 48/80 and antigen, respectively, during "pre-incubation" on histamine release subsequently induced by *the same histamine releaser in the presence of glucose under oxygen* (XIV) or nitrogen (XV) was also investigated. In these experiments the lung tissue was also incubated twice for 35 min; between incubations it was washed as described above. The histamine content of both incubation fluids was assayed. The histamine release from lung tissue incubated in this way (XIV and XV) was compared with the release obtained from lung tissue incubated identically but without exposure to the histamine releaser during "pre-incubation" (XI and XIII). In some experiments where the second incubation was performed under oxygen in the presence of glucose further comparisons were made with lung tissue identically incubated but exposed to the histamine releaser only during "pre-incubation" (XVI) as well as with lung tissue not exposed to the histamine releaser during either incubation (spontaneous histamine release, XVII).

The washing fluid used between incubations was discarded except in a few experiments in which it was assayed for histamine. The histamine value never exceeded 2 % of the total histamine content despite the fact that the lung tissue had been exposed

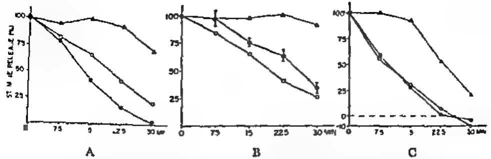

Fig 1 A B and C Effect of pre incubation of rat lung tissue (under nitrogen without glucose) for $7\frac{1}{2}$, 15, $22\frac{1}{2}$ and 30 min on the histamine release subsequently elicited by *Ascaris* extract (1 A) compound 48/80 (1 B) and antigen (1 C) under oxygen with and without glucose and under nitrogen with glucose The results of 10 separate experiments are shown Abscissa = time of pre incubation Histamine release computed in % of histamine release obtained in each experiment without pre incubation Spontaneous histamine release deducted from all values

Incubation procedures

▲—▲—▲ = 0 $7\frac{1}{2}$ 15 $22\frac{1}{2}$ or 30 min N_2 + 15 min glucose O + 20 min hist rel O_2

○—○—○ = 0 $7\frac{1}{2}$ 15 $22\frac{1}{2}$ or 30 min N_2 + 15 min O_2 + 20 min hist rel O_2

●—●—● = 0 $7\frac{1}{2}$ 15 $22\frac{1}{2}$ or 30 min N_2 + 15 min glucose N + 20 min hist rel N_2

I—I—I = range of single tests from 2 identically performed experiments

to compound 48/80 or antigen during pre incubation The time required for the washing procedure was 15 to 30 min depending on the number of samples taken For the same reason the time required for preparation of the lung tissue prior to incubation ranged from $1\frac{1}{2}$ to 3 hours

After withdrawal of the incubation fluids their pH was determined electrometrically The remaining histamine of the lung tissue was extracted as described previously (Diamant 1961 b) Histamine was assayed on atropinized guinea pig ileum Unless otherwise stated the spontaneous histamine release has been deducted from all values

In each experiment a control under nitrogen in the absence of glucose was included (IV) in order to ascertain that anoxic conditions had been attained and inhibited histamine release

Results

(1) *Histamine release from rat lung tissue pre exposed to nitrogen or oxygen*

Fig 1 shows the effect of $7\frac{1}{2}$ to 30 min pre incubation on the release of histamine from rat lung tissue subsequently induced by *Ascaris* extract (Fig 1 A), compound 48/80 (Fig 1 B) or antigen (Fig 1 C) With all three releasers the histamine release was comparatively less sensitive to the inhibitory effect of pre incubation when elicited under oxygen in the presence of glucose (V) This was in comparison to the histamine release induced under oxygen in the absence of glucose (VI) as well as under nitrogen in the presence of glucose (VII) The histamine release decreased successively with duration of pre incubation it started earlier and was more pronounced in the two latter reactions (VI and VII) than in the former (V)

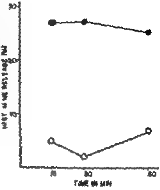

Fig 2 Effect of pre-exposure of rat lung tissue to nitrogen and glucose on histamine release induced by compound 48/80 with and without 30 min pre incubation (under nitrogen without glucose) Abscissa = incubation time under nitrogen with glucose prior to addition of compound 48/80 Histamine release computed in of total histamine content Spontaneous histamine release deducted from all values

Incubation procedures
O—O—O = 30 min N + 15 30 or 60 min glucose N + 20 min 48/80 N
●—●—● = 15 30 or 60 min glucose N + 20 min 48/80 N

In order to investigate whether glucose free pre exposure to oxygen inhibited histamine release subsequently elicited by compound 48/80 under nitrogen in the presence of glucose rat lung tissue was pre exposed to oxygen in a glucose free medium for 30 and 60 min Oxygen was then replaced by nitrogen with concurrent addition of glucose (XVIII) Without pre exposure to oxygen (III) 22 % of the total histamine was released the corresponding figure after glucose free pre-exposure to oxygen for 30 min was 22 % and after 60 min 21 % (mean of double tests from 1 exp) Pre exposure to oxygen in the absence of glucose up to 60 min evidently retained the sensitivity of the lung tissue to compound 48/80

To study the effect of pre exposure to nitrogen in a glucose containing medium one series of rat lung samples was exposed to nitrogen and glucose for varying periods before addition of compound 48/80 (XIX) The results (Fig 2) show that when pre exposure to nitrogen takes place in the presence of glucose for up to at least 60 min the sensitivity of the lung tissue to compound 48/80 is retained It is notable that no increase in histamine release was recorded after 30 and 60 min nitrogenation in the presence of glucose as compared to that after 15 min This indicates that optimal conditions for histamine release in a nitrogenated medium were reached within 15 min of contact of the lung tissue with glucose

In the same experiment (Fig 2) another series of rat lung samples was first pre incubated (in a glucose free nitrogenated medium) for 30 min After addition of glucose incubation was continued under nitrogen for 15 30 and 60 min respectively before the addition of compound 48/80 (XX) The results indicate that the depressant action exerted by pre incubation for 30 min on the histamine release cannot in fact be overcome by prolonged contact with glucose (up to 60 min) during continuous nitrogenation prior to the addition of compound 48/80

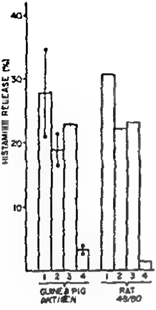

Fig 3

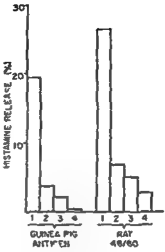

Fig 4

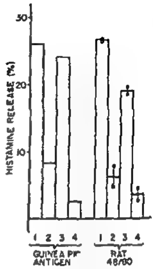

Fig 5

Fig 3 Effect of washing the lung tissue after 35 min pre incubation (under nitrogen without glucose) on histamine release subsequently induced under oxygen in presence of glucose by antigen and compound 48/80 from guinea pig and rat lung tissue respectively Filled and open circles represent individual values in separate experiments Histamine release shown in sample 3 refers to the reincubation period Histamine release computed in % of total histamine content at the time of incubation Spontaneous histamine release deducted from all values

Incubation procedures
1 15 min glucose O_2 → 20 min hist rel O_2
2 35 min N_2 → 15 min glucose O_2 → 20 min hist rel O_2
3 35 min N_2 || 15 min glucose O_2 → 20 min hist rel O_2
4 15 min N_2 → 20 min hist rel N_2

Fig 4 Effect of washing the lung tissue after 35 min pre incubation (under nitrogen without glucose) on histamine release subsequently induced under oxygen without glucose by antigen and compound 48/80 from guinea pig and rat lung tissue respectively Histamine release shown in sample 3 refers to the reincubation period Histamine release computed in % of total histamine content at the time of incubation Spontaneous histamine release deducted from all values.

Incubation procedures
1 15 min O_2 → 20 min hist rel O_2
2 35 min N_2 → 15 min O_2 → 20 min hist rel O_2
3 35 min N_2 || 15 min O_2 → 20 min hist rel O_2
4 15 min N_2 → 20 min hist rel N_2

Fig 5 Effect of washing the lung tissue after 35 min pre incubation (under nitrogen without glucose) on histamine release subsequently induced under nitrogen in presence of glucose by antigen and compound 48/80 from guinea pig and rat lung tissue respectively Filled and open circles represent individual values in separate experiments Histamine release shown in sample 3 refer to the reincubation period Histamine release computed in % of total histamine content at the time of incubation Spontaneous histamine release deducted from all values

Incubation procedures
1 15 min glucose N_2 → 20 min hist rel N_2
2 35 min N_2 → 15 min glucose N_2 → 20 min hist rel N_2
3 35 min N_2 || 15 min glucose N_2 → 20 min hist rel N_2
4 15 min N_2 → 20 min hist rel N_2

(2) *Effect of washing the lung tissue after pre incubation on subsequently elicited histamine release*

Histamine release was induced under oxygen in the presence (Fig 3) or absence (Fig 4) of glucose or under nitrogen in the presence of glucose (Fig 5) Each experiment shown in Figs 3 4 and 5 represents the histamine release from 4 samples of lung tissue which were incubated as given below the figures

The marked inhibition of histamine release caused by anoxic glucose free experimental conditions (IV) is evident from sample 4 in Figs 3 4 and 5 Sample 4 therefore served as a control that anoxic conditions had been attained in each experiment

A comparison between the histamine release from lung tissue incubated without pre incubation (sample 1 in Figs 3 4 and 5) and that from lung samples exposed to 35 min pre incubation but not to washing (sample 2 in Figs 3 4 and 5) again emphasizes that histamine release from rat lung tissue was less inhibited by pre incubation when induced by compound 48/80 under oxygen with glucose (VIII) than under nitrogen with glucose (X) or under oxygen without glucose (IX) Moreover similar effects are evident with respect to antigen induced histamine release from guinea pig lung tissue

The histamine release from lung samples which had been washed with 10 ml of phosphate medium after the pre incubation period (sample 3 in Figs 3 4 and 5) did not show any pronounced changes when reincubation was performed under oxygen in the presence or absence of glucose (Figs 3 and 4) as compared with the histamine release from lung tissue identically incubated except for the washing procedure (sample 2 in Figs 3 and 4) In contrast when reincubation was performed under nitrogen in the presence of glucose washing the lung tissue after pre incubation had a markedly activating effect on the histamine release in both histamine releasing systems (Fig 5)

(3) *Histamine release from lung tissue once exposed to the histamine releaser during pre incubation*

It is apparent from Figs 3 4 and 5 that in order to evaluate the effect of pre exposing lung tissue to histamine releasing agents under conditions inhibitory to histamine release *i e* during pre incubation on the histamine release subsequently induced by the same histamine releaser reincubation (after washing) had to be performed in the presence of glucose under oxygen or nitrogen This effect was investigated on anaphylactic histamine release from guinea pig lung tissue and on compound 48/80 induced histamine release from rat lung tissue as shown in Tables II and III where reincubation of the lung tissue was performed under oxygen in the presence of glucose The sum of the histamine released during the two incubations was determined and expressed as a percentage of the total histamine content of the lung tissue The spontaneous histamine release from both incubations is included in this value

Table II Effect of pre-exposure of guinea pig and rat lung tissue to antigen and compound 48/80, respectively under glucose free anoxic conditions ('pre incubation') on the subsequent histamine release induced under oxygen in the presence of glucose by the same histamine releaser 2 experiments (single tests) with each releaser are shown Histamine release given as µg histamine base from 1 g of lung tissue Spontaneous histamine release included $\frac{a}{b} \times 100$ = total histamine released during both incubations in % of total histamine content

Histamine release

	Antigen — Guinea-pig lung tissue			
	Experiment 1		Experiment 2	
	A	B	A	B
Pre incubation (N_2)	Without antigen 0.12	With antigen 0.77	Without antigen 0.12	With antigen 0.26
Reincubation (O_2+glucose)	With antigen 3.95	With antigen 0.70	With antigen 2.18	With antigen 0.58
Total histamine released (a)	4.07	1.47	2.30	0.84
Total histamine content (b)	16.8	17.1	9.59	9.38
$\frac{a}{b} \times 100$	24.2	8.6	24.0	9.0
	Compound 48/80 — Rat lung tissue			
	Experiment 3		Experiment 4	
	A	B	A	B
Pre incubation (N_2)	Without 48/80 0.40	With 48/80 0.47	Without 48/80 0.18	With 48/80 0.70
Reincubation (O_2+glucose)	With 48/80 1.32	With 48/80 0.64	With 48/80 1.56	With 48/80 0.67
Total histamine released (a)	1.72	1.11	1.74	1.37
Total histamine content (b)	4.83	4.62	4.26	4.50
$\frac{a}{b} \times 100$	35.2	24.0	40.8	30.4

Incubation procedures
A. 35 min N_2 ‖ 15 min glucose O_2 + 20 min hist rel O_2
B 15 min N_2 + 20 min hist rel N_2 ‖ 15 min glucose O_2 + 20 min hist. rel O_2

In Table II a comparison is shown between the histamine release obtained from lung tissue exposed to the histamine releaser during both incubations (XIV B in Table II) and that found on exposure during the reincubation period only (XI A in Table II) When antigen was added to guinea pig lung tissue during the reincubation period only 24.1 % of the total histamine content was released whereas 8.6 % was found when it was exposed to antigen

Table III Effect of pre-exposure of guinea pig and rat lung tissue to antigen and compound 48/80 respectively under glucose free anoxic conditions (pre incubation) on the subsequent histamine release induced under oxygen in presence of glucose by the same histamine releaser 1 experiment (double tests) with each histamine releaser is shown Histamine release given in µg histamine base from 1 g of lung tissue Spontaneous histamine release included $\frac{a}{b} \times 100$ = total histamine released during both incubations in of total histamine content

Histamine release

	Antigen — Guinea pig lung tissue			
	A	B	C	D
Pre incubation (N)	Without antigen 0.19 0.19	With antigen 0.44 0.42	With antigen 0.42 0.43	Without antigen 0.20
Reincubation (O + glucose)	With antigen 2.75 2.82	With antigen 0.55 0.6?	Without antigen 0.39 0.48	Without antigen 0.44
Total histamine released (a)	2.94 3.01	0.99 1.04	0.81 0.91	0.64
Total histamine content (b)	13.3 13.2	14.3 13.7	12.3 12.3	12.4
$\frac{a}{b} \times 100$	22.1 22.8	6.9 6.9	6.6 7.4	5.1
Mean of double tests	22.5	6.9	7.0	
	Compound 48/80 — Rat lung tissue			
	A	B	C	D
Pre incubation (N)	Without 48/80 0.42 0.41	With 48/80 0.93 0.68	With 48/80 0.68 1.00	Without 48/80 0.42 0.45
Reincubation (O + glucose)	With 48/80 3.06 2.79	With 48/80 1.11 1.13	Without 48/80 0.85 0.83	Without 48/80 0.38 0.36
Total histamine released (a)	3.48 3.20	2.04 1.81	1.53 1.83	0.80 0.81
Total histamine content (b)	7.87 7.64	7.88 7.55	7.39 7.85	7.29 7.42
$\frac{a}{b} \times 100$	44.2 41.9	25.9 23.8	20.7 23.3	11.0 10.9
Mean of double tests	43.1	24.9	22.0	10.5

Incubation procedures
A 35 min N_2 ‖ 15 min glucose O_2 + 20 min hist rel. O_2
B 15 min N_2 + 20 min hist rel N_2 ‖ 15 min glucose O_2 + 20 min hist rel. O_2
C 15 min N_2 + 20 min hist rel. N_2 ‖ 35 min glucose O_2
D 35 min N_2 ‖ 35 min glucose O_2

during both incubations (mean of exp 1 and 2 Table II) The corresponding values for compound 48/80 induced histamine release from rat lung tissue amounted to 38.0 % and 27.2 % (mean of exp 3 and 4 Table II)

In additional experiments, reincubation was performed under nitrogen in the presence of glucose The histamine release from guinea pig lung tissue exposed to antigen during the reincubation period only (XIII) amounted to 26 4 % of the total histamine content, whereas when antigen was present during both incubations (XV) 13 0 % was released (1 exp) The corresponding values for compound 48/80-induced histamine release from rat lung tissue were 27 7 % and 20 7 % (mean of 2 exp)

It is evident from these experiments that the total histamine release during the two incubations was less after the lung tissue had been exposed to the histamine releaser during both incubations, as compared with lung tissue exposed only during the reincubation period In order to obtain more quantitative data on this effect lung samples were exposed in pairs to the histamine releaser during the reincubation period only (XI A in Table III) during both incubations (XIV B in Table III) and during the pre incubation period only (XVI, C in Table III) The controls (D in Table III) denote the sum of the spontaneous histamine release during both incubations (XVII) The histamine release from lung samples exposed to the histamine releaser during both incubations (B) was almost identical to that obtained from lung samples exposed to the histamine releaser during pre incubation only (C) This applied to both histamine releasing systems shown in Table III Consequently, it seems justified to conclude that once guinea pig and rat lung tissue has been exposed to antigen and compound 48/80, respectively under conditions inhibitory to histamine release ("pre incubation) the tissue be almost completely insensitive to a second exposure to the same histamine releasing agent It is further evident (Tables II and III) that this desensitizing effect was more apparent in the case of antigen and guinea pig lung tissue than in the case of compound 48/80 and rat lung tissue As can be noted in Table III this might be attributed to the fact that the histamine release obtained after administration of compound 48 80 during "pre incubation alone (C) markedly exceeded the spontaneous histamine release (D)

Discussion

The present investigation gives further support to the view that histamine release *in vitro* induced by *Ascaris* extract compound 48/80 and antigen from rat lung tissue and by antigen from guinea pig lung tissue are reactions depending on adequate metabolism for energy production

It is evident from the present results as well as from earlier publications (Diamant 1961 a b Diamant and Uvnäs 1961) that the histamine releasing reactions in question occur in a substrate free medium as long as oxygen is available This is despite the fact that the procedure for preparation of the lung tissue involved frequent washing with a substrate free phosphate buffer for $1^{1}/_{2}$ to 3 hours prior to incubation. During this time it is highly probable that

the composition of the endogenous energy yielding substrates had undergone marked changes. GEY (1956) noted about 50 % decrease in the glucose content of rat lung tissue 3 min after death of the animals. FISCHER and WILLIAMSON (1961) measured the glycogen content of nutrient free perfused rat hearts and found that glycogen disappeared after the first hour of perfusion. During this time its metabolism would account for 20 to 30 % of the oxygen consumed. Since the respiration of the perfused rat hearts mostly remained unchanged during the first hour they concluded that endogenous substrates other than carbohydrates were used for oxidation. It is so far unknown whether similar drastic changes in the carbohydrate stores occur in lung tissue during the preparation procedure — so that it has to rely on oxidation of non-carbohydrate substrates to fill the energy requirement — or whether the changes are more of a quantitative nature. The enhancing effect of glucose on the histamine release elicited under oxygen suggests however that whatever changes may have occurred in the endogenous substrates during the preparation procedure oxidation of the remaining substrates does not yield optimal energy for the histamine releasing reactions.

The interruption of respiration by anaerobic conditions limits metabolism to glycolytic breakdown of carbohydrates with production of chemical energy (adenosine triphosphate = ATP) via the Embden Meyerhof pathway. When, under anaerobic conditions carbohydrate stores have been depleted there is no further source of energy unless exogenous glucose is supplied. The results of the present investigation as well as of earlier ones seem to be compatible with the aforegoing facts since they have shown that histamine release is inhibited under nitrogen in the absence of glucose in the incubation medium. If however glucose is supplied from the beginning of the incubation under nitrogen this inhibition is counteracted. Since in those experiments performed in the absence of glucose the incubation procedure consisted of 15 min incubation under nitrogen before addition of the histamine releasers this procedure probably depleted the carbohydrate stores even further as shown in frog muscle (RONZONI and EHRENFEST 1936) and in dog and rat hearts (MICHAL *et al* 1959 FISCHER and WILLIAMSON 1961 CORNBLATH MORGAN and RANDLE 1961). Furthermore the ATP possibly present in the lung tissue after the preparation procedure most probably disappeared during this 15 min period of anoxia in the absence of glucose. This has repeatedly been shown to be the case in muscle tissues exposed to nitrogen (RONZONI and EHRENFEST 1936 GREINER 1952 FURCHGOTT and GUBAREFF 1958 MICHAL *et al* 1959).

PARROT (1942) reported that pre-exposure of guinea pig lung tissue to nitrogen did not induce changes in the subsequent histamine release when caused by antigen in the presence of oxygen. Since these experiments were performed in Tyrode solution which generally contains glucose the results are in agreement with the present findings. On the other hand my results do not agree with those of MOUSSATCHE and PROVOUST DANON (1960). They found, when using

a glucose free incubation medium (MOUSSATCHÉ 1961) that pre-exposure of sensitized guinea pig lung tissue to nitrogen for 45 min did not decrease the histamine release when the lung tissue was subsequently exposed to antigen under oxygen.

It is apparent from the present results that pre-exposure of lung tissue to nitrogen in the absence of glucose ("pre-incubation") had essentially the same effect on histamine release induced by the various agents tested. Thus when incubation of the lung tissue was continued under oxygen in the presence of glucose, less inhibition was noted than when incubation was continued under nitrogen in the presence of glucose or under oxygen in its absence. If it is assumed that "pre-incubation" of the lung tissue induced a progressive deficit of ATP, as well as exhaustion of the endogenous carbohydrate stores (as shown in muscle tissues), the presence of glucose under continued anoxic incubation did not, apparently, restore this deficit by its anaerobic breakdown — as judged by the impaired histamine release. One reason might be that lack of ATP primarily inhibited reactions involved in initiating glycolysis. This was suggested by ELLIOTT and ROSENFELD (1958) to be the cause of the rapid loss of glycolytic activity in rat brain slices, noted after pre-incubation in the absence of glucose and oxygen. Similar conclusions were drawn by MORGAN, RANDLE and REGEN (1959) from experiments with rat heart tissue which after anaerobic incubation without glucose showed impaired uptake and utilization of glucose on continued anaerobic incubation with the sugar. Another possible explanation is that the mechanism involved in the utilization of ATP became impaired by "pre-incubation". The marked activation of histamine release under nitrogen in the presence of glucose caused by washing the lung tissue after 35 min "pre-incubation" suggests that this procedure induced either activation of the utilization mechanism of ATP, or changes in the lung tissue which made it possible to partly restore the ATP content by breakdown of glucose via the Emden-Meyerhof pathway. Since during the washing procedure, the lung tissue was in contact with air for 15 to 30 min before reincubation, this might be an additional factor influencing the subsequent anaerobic utilization of glucose, as discussed by QUASTEL and WHEATLEY (1937) and ELLIOTT and ROSENFELD (1958) on the basis of experiments on glycolysis in brain slices.

The histamine release was also markedly inhibited by "pre-incubation" when incubation was continued and histamine release elicited under oxygen without glucose, but here washing had no activating effect. This might be explained by postulating that "pre-incubation" of the lung tissue caused — in addition to a deficit of ATP and exhaustion of carbohydrate stores — inhibition of the utilization of substrates other than carbohydrates, and that these changes could not be reversed by washing the lung tissue.

The histamine release elicited under oxygen in the presence of glucose was comparatively little affected by "pre-incubation". This suggests that when

glucose was metabolized in the presence of oxygen the deficit of ATP could be replaced, or its utilization mechanism restored at least partly even without washing the lung tissue

The present investigation has further shown that pre exposure of rat lung tissue to oxygen in the absence of glucose as well as to nitrogen in the presence of glucose for up to 60 min did not affect the subsequent histamine release induced under nitrogen in the presence of glucose by compound 48/80 This implies that the postulated deficit of ATP to an extent affecting the histamine releasing reactions occurs only under glucose free anoxic pre-exposure

This hypothetical interpretation of the results does not take into account several factors that could be influenced by anoxic conditions and which would thereby lead to changes in formation or utilization of ATP and consequently to inhibition of the histamine release Examples of such factors are changes in adenosine triphosphatase activity lack of phosphate acceptors changes of pyridine nucleotides and accumulation of inhibitors to reactions involved in utilization of glucose and endogenous substrates If however histamine release from lung tissue exposed to the various metabolic situations can be shown to reflect changes in either content or utilization of ATP the present results will substantiate the importance of the metabolic reactions that have taken place in the lung tissue before it is exposed to the histamine releaser

In view of the parallelism between histamine release from tissues and isolated rat mast cells and degranulation of mast cells of rat peritoneum with respect to factors influencing metabolism (metabolic inhibitors anoxia and glucose) UVNÄS (1961 b) concluded that the energy requiring release of histamine depends on the metabolic reactions occurring in the mast cells It has not so far been possible to investigate to what extent the present results might reflect changes in the metabolic situation of the mast cells of the lung tissue since histamine release from isolated rat mast cells is not affected by anoxia in the absence of glucose (UVNÄS and THON 1961 a MORAN UVNÄS and WESTERHOLM 1961) At first sight this may appear inconsistent with the importance of the metabolic reactions occurring in mast cells Since albumin is necessary for histamine to be released from isolated rat mast cells (UVNÄS and THON 1961 a), it was suggested that the albumin might render the mast cells insensitive to anoxia (MORAN UVNÄS and WESTERHOLM 1961) The importance of the metabolic reactions occurring in isolated rat mast cells is however evident from the finding that histamine release induced by *Cyanea capillata* and by compound 48/80 was inhibited by dinitrophenol whereas this inhibition was abolished in the presence of glucose (UVNÄS 1960, MORAN UVNÄS and WESTERHOLM 1961)

The present investigation has shown that guinea pig and rat lung tissue pre-exposed to antigen and compound 48/80 respectively under conditions inhibitory to histamine release (*i e* nitrogen in the absence of glucose) became insensitive to a second exposure to the same histamine releaser This

applied although (after washing) re exposure took place under oxygen in the presence of glucose It was shown that the desensitization of the lung tissue was not due to the anoxic glucose free pre exposure itself In all experiments this desensitizing effect was less marked in reactions with compound 48/80 and rat lung tissue than in reactions with antigen and guinea pig lung tissue The reason for this difference cannot be finally evaluated from the present results These observations are, however interesting in view of those made by MONGAR and SCHILD (1957) They found that when sensitized guinea pig lung tissue was exposed to antigen in the presence of phenol, histamine release was inhibited When after removal of the phenol, the lung tissue was re exposed to antigen there was still no histamine release Furthermore, UVNAS and THON (1961 b) noted that isolated rat mast cells exposed to compound 48/80 at pH 5 did not release histamine When the cells were reincubated at pH 7, re exposure to compound 48/80 still failed to elicit histamine release In controls, pre exposure of mast cells at pH 5 without compound 48/80 did not inhibit subsequent histamine release from them, when they were reincubated at pH 7 and exposed to compound 48/80

Consequently it is suggested that under anoxic glucose free conditions the histamine releasing agents tested initiate reactions in the mast cells of the lung tissue which should lead to histamine release but that are stopped at some subsequent critical step where high energy compounds are needed The nature of these reactions is unknown but their possible role is indicated by the fact that the histamine release was inhibited when the lung tissue was re exposed to the same histamine releaser under conditions normally favouring formation of high energy compounds (i e oxygen in the presence of glucose) possible explanation might be that during the reincubation period, some active sites on the mast cells were already occupied by the histamine releaser On the basis of their studies on sensitized guinea pig lung tissue MONGAR and SCHILD (1957) postulated that antigen induced histamine release depends on the activation of a short lived enzyme system, which catalyzes reactions leading to histamine release HOGBERG and UVNAS (1957 1960) UVNAS and THON (1961 a) and UVNAS (1961 b) suggested that a lytic enzyme triggering histamine release was activated by compound 48/80 and antigen antibody reaction This was evidenced by the parallelism between the effect of various enzyme inhibitors on the degranulation of mast cells in rat peritoneum as well as on the histamine release from isolated rat mast cells when induced by compound 48/80 antigen antibody reaction and phosphatidase A The desensitization of the lung tissue noted in the present investigation could therefore be explained on the assumption that in the absence of energy the histamine releasing agents initiated reactions which irreversibly interfered with the activation mechanism either of the enzyme or of some reaction triggered by it

Still other interpretations of the results must be considered The histamine releasers — when affecting the lung tissue under conditions inhibitory to hista

mine release — may induce changes in the mast cell membrane thereby causing impairment either of the subsequent uptake and utilization of glucose or possibly of some transport mechanism so far unknown but a prerequisite for histamine release This possibility is supported by the observations of BICKIS QUASTEL and VAS (1959) who noted changes in transport phenomena in Ehrlich ascites tumour cells after addition of specific antisera even in doses which did not produce morphological changes in the cells

Whatever the reason may be for the desensitizing effect observed in the present investigation the results suggest that the energy requiring step of the reactions leading to histamine release must, actually be inherent in some reaction occurring after the coupling of the histamine releasing agents to active sites in the lung tissue

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5-Hydroxytryptamine in the Schultz-Dale Reaction

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Abstract

BOREUS L O and B WESTERHOLM *5-Hydroxytryptamine in the Schultz Dale reaction* Acta physiol scand 1962 *56* 17—25 — When anaphylactic smooth muscle contraction (Schultz Dale reaction) was produced in ileum and uterus of the rat and guinea pig it led to release of a substance which had contractile effect on guinea pig ileum but not on highly 5 HT sensitive rat uterus Following the Schultz Dale reaction the tissues showed no significant changes in 5 HT or histamine content when compared to controls from the same animals However the individual variation in amine content was high Sensitized rat and guinea pig tissues when incubated with antigen exhibited no measurable release of 5 HT A decrease of the very low 5 HT content of rat uterus was however noted when uteri from many animals were pooled and incubated Histamine was liberated from uterus of both rat and guinea pig but not from ileum It is concluded that 5 HT is probably not involved in the Schultz Dale reaction in the guinea pig though it may be partly responsible for the anaphylactic contraction of rat uterus

It has been suggested that release of 5 hydroxytryptamine (5 HT) is instrumental in the anaphylactic contraction of guinea pig smooth muscle, *i e*, the Schultz Dale reaction (GEIGER HILL and THOMPSON 1956 GEIGER and ALPERS 1959) This conclusion was founded on experiments in which inhibitors of 5 HT had been added to the bath prior to antigen Attempts to demonstrate directly the release of 5 HT, however have not proved successful (FINK and GARDNER 1958)

All the above investigators used ileum as test tissue It has been shown that uterus releases more histamine than does ileum when incubated with antigen (SCHILD 1939) Considerably more smooth muscle stimulating sub-

stance, in terms of histamine, was released from uterus than from ileum during the Schultz Dale contraction (BORÉUS 1961)

In this study therefore the possible release of 5 HT from these smooth muscle tissues in guinea pig and rat after antigen contact was studied via both biological and chemical assay In some experiments the histamine release under the same conditions was also studied, since it would seem that the mechanism of release of these two amines involves a similar mechanism (MORAN, UVNÄS and WESTERHOLM 1962)

Methods

Female virginal rats (Wistar strain) weighing about 100 g and female virginal guinea pigs weighing about 300 g were used

Rats were sensitized by injecting 100 mg of crystalline egg albumin and 1 ml pertussis vaccine (2×10^{10} organisms/ml) subcutaneously Guinea pigs were sensitized with 100 mg of the same egg albumin subcutaneously and 3 days later with the same dose intraperitoneally The experiments were performed 2—5 weeks later, when the animals were killed by a blow on the head and exsanguinated by cutting the neck The tissues were removed and kept in the suspending medium until use

Antigen induced release of 5 HT and histamine from the sensitized tissues was investigated in two ways

Schultz Dale technique (with biological 5-HT assay) A segment of sensitized ileum uterus or spleen was suspended at 37 C in a 5 ml aerated bath washed by overflow The contractions of sensitized ileum or uterus (the 'SD segment) were recorded by a frontal lever on a kymograph drum During the experiment a contiguous tissue segment from the same animal was immersed in a beaker with the medium at 37° C and used as a control

An indicator segment of uterus from a non sensitized rat in estrus phase (rats injected with estradiol benzoate 5 mg/kg 24—36 hours before use) or a segment from a non sensitized guinea pig were suspended in the same bath in order to reveal any 5-HT or histamine liberated from the sensitized tissue by the antigen The indicator segment was attached to another frontal lever A similar technique has been used by DWORETZKY (1959)

In the 5-HT experiments the following medium as reported by ENGELHARDT and SCHWABE (1960) was used NaCl $1\,54 \times 10^{-1}$M KCl $5\,6 \times 10^{-3}$M $CaCl_2$ $5\,0 \times 10^{-4}$M $NaHCO_3$ $5\,0 \times 10^{-3}$M dextrose $2\,8 \times 10^{-3}$M and atropine sulphate 1.5×10^{-7}M In the other experiments the suspending medium was Tyrode solution containing dextrose ($5\,\text{[illegible]} \times 10^{-3}$M) and atropine sulphate ($1\,5 \times 10^{-6}$M)

When dose response curves for 5-HT and histamine had been obtained antigen to a final concentration of 0 4 mg/ml was added and allowed to remain in the bath for 3 minutes (higher antigen concentrations could not be used because of scum formation in the bath) Any contraction of the indicator segment during this period was estimated in terms of 5-HT and histamine respectively At the end of the 3 minute period the antigen was washed out. The sensitized organ was taken out of the bath weighed and immersed in 0 1 N HCl for later 5-HT and histamine assay The control tissue was similarly treated

The volumes of drugs added to the bath (5 HT creatinine sulphate histamine dihydrochloride or egg albumin dissolved in appropriate medium) were 0 2 ml or less

Incubation technique (with chemical 5-HT assay) Ileum uterus spleen and — in a few experiments — abdominal skin were pooled from a group of animals The tissues were

Table I Histamine and 5-HT content (means and standard errors) of ileum uterus spleen and skin of the rat and guinea pig Figures within brackets denote numbers of determinations made

	Histamine (μg/g tissue)		5-HT (μg/g tissue)	
	Rat	Guinea pig	Rat	Guinea pig
Ileum	7 9 ± 1 1 (28)	10 2 ± 1 6 (9)	1 9 ± 0 3 (27)	3 6 ± 0 8 (9)
Uterus	1 2 ± 0 2 (10) < 0 15 (7)	5 7 ± 1 6 (9)	0 15 ± 0 05 [a](7)	< 0 003 (2)
Spleen	1 0 ± 0 2 (25)	2 9 ± 0 7 (12)	2 3 ± 0 5 (24)	3 7 ± 0 5 (12)
Skin	10 5 ± 1 2 (24)	3 6 ± 0 6 (8)	0 8 ± 0 1 (24)	< 0 01 (8)

Pooled groups (each group 5—15 animals) [a] Pooled groups (each group 15 animals)

cut and put into 25 ml beakers containing 3 ml of a buffered solution (pH 7 2—7 4) containing NaCl 1 54 × 10^{-1}M KCl 2 7 × 10^{-3}M $CaCl_2$ 9 0 × 10^{-4}M 10 per cent Sorensen phosphate buffer (Na_2HPO_4 2 H_2O 3 0 × 10^{-3}M KH_2PO_4 3 5 × 10^{-} M) and dextrose 5 6 × 10^{-3}M The beakers were slowly rocked in a water bath at 37 C. Oxygen was introduced through cannulae in the stoppers of the beakers After an interval of 10 minutes for temperature equilibration antigen (final concentration 1 mg/ml) was added All incubations were duplicate After 30 min the supernatants were poured into tubes for 5 HT and histamine assay The tissues were immersed in 0 1 N HCl for later 5 HT and histamine determination

5-HT and histamine assay Tissues for 5-HT and histamine determination were homogenized in 0 1 N HCl The tissue extracts as well as the supernatants were kept frozen until assay

5 HT in tissues and supernatants was determined spectrophotofluorimetrically by the method of Bogdanski *et al* (1956) Histamine was tested after neutralization of the samples on atropinized (atropine sulphate 1 5 × 10^{-6}M) guinea pig ileum Its specificity was demonstrated by mepyramine block (5 0 × 10^{-7}M)

All 5-HT and histamine values are expressed as the free base

Results

5-HT and histamine content of rat and guinea pig tissues

During the course of the experiments we made several determinations of the 5 HT and histamine content in different organs of the rat and guinea pig These estimations are summarized in Table I which shows the amine content of ileum, uterus spleen and skin. It will be seen that the individual variation was high for all tissues The 5 HT content was very low in rat uterus In guinea pig uterus it was still lower not even when organs from fifteen animals were pooled and extracted could 5 HT be demonstrated Nor was it possible to detect any 5 HT in guinea pig skin

Table II Antigen induced release of histamine and 5-HT (means and standard errors) from ileum and uterus (SD reaction) and spleen of the sensitized rat Final antigen concentration 0 4 mg/ml. Exposure time 3 min Figures within brackets denote numbers of experiments made

	Liberated contracting substance (μg/g tissue) in terms of histamine	Liberated contracting substance (μg/g tissue) in terms of 5-HT	Histamine content (μg/g tissue) after experiment		5-HT content (μg/g tissue) after experiment	
			Control strip	SD strip	Control strip	SD strip
Ileum	>(0.01–0.1) (4)	<(0.0007–0.004) (7)	6.7 ± 1.2	6.6 ± 1.1 (11)	2.3 ± 0.6	2.2 ± 0.5 (11)
Uterus	>(0.01–0.1) (4)	<(0.0008–0.003) (7)	Not measurable (11)		Not measurable (11)	
Spleen	>(0.01–0.02) (4)	<(0.0004–0.007) (6)	1.3 ± 0.3	0.9 ± 0.2 (10)	2.9 ± 1.0	3.1 ± 1.0 (10)

Release of 5-HT and histamine from rat and guinea pig tissues in vitro

(1) *Schultz Dale experiments with rat tissues* (Table II) Addition of antigen to the sensitized ileum or uterus caused a Schultz Dale reaction i e, a rapid contraction of the organ starting within 10—20 sec, reaching its maximum within 30 sec and followed by relaxation to the original length after 2 to 6 min. Sensitization of the animals was thereby demonstrated No contraction was elicited by the same egg albumin in non sensitized rat ileum or uterus) During the Schultz Dale reaction a smooth muscle stimulating principle was released causing contraction of an indicator segment of guinea pig ileum, sensitive to a concentration of 1 μg 5 HT per ml and 1 ng histamine per ml. A contracting substance was also released from sensitized spleen Contraction of the indicator segment began a few seconds after the Schultz Dale contraction and rose gradually to its maximum. The segment relaxed immediately when washed 3 min later

To determine whether 5-HT was a constituent of the smooth muscle stimulating principle the indicator guinea pig ileum was replaced by non sensitized rat uterus sensitive to a concentration of 0 2 ng 5-HT per ml and 0 2 μg histamine per ml. When antigen was added to the bath no release of contracting substance from the sensitized tissues could be demonstrated in spite of the highly 5-HT sensitive indicator organ (Table II) The results indicate that 5-HT is not released into the bath during the Schultz Dale reaction. This finding is corroborated by the fact that the 5-HT content of the antigen treated ileum and spleen was not significantly lower than that of control segments from the same animals The 5-HT content of uterus could not be determined because of the small amount of available tissue from any one

Table III Antigen induced release of histamine and 5 HT (means and standard errors) from ileum and uterus (SD reaction) and spleen of the sensitized guinea pig Final antigen concentration 0 4 mg/ml Exposure time 3 min Figures within brackets denote numbers of experiments made

	Liberated contracting substance (μg/g tissue) in terms of 5-HT	Histamine content (μg/g tissue) after experiment		5-HT content (μg/g tissue) after experiment	
		Control strip	SD strip	Control strip	SD strip
Ileum	<(0 0004–0 003)	12 9 ± 2 5	11 8 ± 1 7	3 5 ± 1 1	3 9 ± 1 1
	(4)	(4)		(4)	
Uterus	< (0 001–0 00?)	7 2 ± 2 1	5 5 ± 2 0	Not measurable	
	(4)	(4)		(4)	
Spleen	<(0 0006–0 001)	2 8 ± 0 8	2 3 ± 0 4	4 4 ± 0 4	3 9 ± 0 4
	(4)	(4)		(4)	

Table IV Release of histamine and 5-HT (means and standard errors) from sensitized ileum uterus and spleen of the rat following incubation with antigen (1 mg/ml) Incubation time 30 min Figures within brackets denote numbers of experiments made

	Histamine liberation per cent		5 HT liberation	Histamine content (μg/g tissue) after incubation		5-HT content (μg/g tissue) after incubation	
	Control samples	Antigen treated samples		Control samples	Antigen treated samples	Control samples	Antigen treated samples
Ileum	<(0 003–0 2)	<(0 003–0 3)	Not measurable	7 6±1 0	7 6±0 8	0 9±0 2	1 0±0 2
	(18)			(18)		(18)	
Uterus	10 7 ± 4 2	36 8 ± 6 5	Not measurable	1 0±0 1	0.8±0 1	0 17 0 15	*0 09 <0 09
	(9)			(9)			
Spleen	25 5 ± 4 5	40 9 ± 5 6	Not measurable	0 9±0 2	0 8±0 2	1 7±0 4	1 6±0 5
	(14)			(14)		(22)	

Values obtained from uteri pooled from 34 animals
* Values obtained from uteri pooled from 10 animals.

animal Ileum and spleen showed no reduction of the histamine content, while in uterus the content was too small to be measured

(2) *Schultz Dale experiments with guinea pig tissues* (Table III) That a smooth muscle stimulating principle causing contraction of indicator guinea pig ileum is released from guinea pig ileum and uterus during the Schultz Dale

Table V Release of histamine and 5-HT (means and standard errors) from sensitized ileum, uterus and spleen of the guinea pig following incubation with antigen (1 mg/ml). Incubation time 30 min. Figures within brackets denote numbers of experiments made

	Histamine liberation per cent		5-HT liberation	Histamine content ($\mu g/g$ tissue) after incubation		5-HT content ($\mu g/g$ tissue) after incubation	
	Control samples	Antigen treated samples		Control samples	Antigen treated samples	Control samples	Antigen-treated samples
Ileum	< 0.1	< (0.1–0.3)	Not measurable	9.1 ± 0.7	9.9 ± 1.2	3.6 ± 1.4	3.7 ± 1.4
	(6)			(6)		(6)	
Uterus	< (0.1–0.3)	5.8 ± 1.7	Not measurable	6.8 ± 1.8	5.8 ± 1.6	Not measurable	
	(6)			(6)			
Spleen	14.2 ± 4.8	13.9 ± 4.0	Not measurable	3.1 ± 1.0	3.1 ± 1.0	4.6 ± 0.7	4.8 ± 0.6
	(10)			(10)		(10)	

reaction has been shown by BORELS (1961). The following experiments were designed to show whether 5-HT is a constituent of the contracting substance. As in the experiments with rat tissues no release of 5-HT could be demonstrated with the highly sensitive rat uterus, though a typical Schultz Dale contraction occurred in all experiments on ileum and uterus (Table III). Further there was no detectable 5-HT release from spleen. Neither the 5-HT content nor the histamine content was lower in the antigen treated tissues than in the controls. It should be noted, however, that the 5-HT content of uterus was too low to be measured.

(3) *Incubation experiments with rat tissues* (Table IV). Since the results of the Schultz Dale experiments in respect to 5-HT release were negative, the study was extended to *in vitro* incubation experiments in which it was possible to use about ten times as much tissue substrate and to reduce the volume of incubation fluid to 3 ml. Even here, however, no release of 5-HT into the medium could be demonstrated. The residual amounts of 5-HT in tissues incubated with and without antigen were therefore compared. It will be seen from Table IV that there was no significant reduction of the 5-HT content of ileum and spleen. In uterus, on the other hand, antigen caused a reduction of 53 and 60 per cent of the 5-HT content in two experiments with pooled uteri from 34 and 10 rats respectively.

Histamine was shown to be released into the incubation fluid from uterus. From ileum, however, no release of histamine was demonstrable. In the case of spleen, there was some difference in release values between control and antigen treated samples ($P = 0.05$–0.01). However, the spontaneous histamine release was high (25.5 per cent).

(4) *Incubation experiments with guinea pig tissues* (Table V) As in the experiments with rats no 5 HT could be detected in the incubation fluid after addition of antigen Nor was it possible to demonstrate any reduction of the 5 HT content in ileum and spleen. In uterus — both control and antigen treated samples — the 5 HT content was too low to be measured Histamine was released from uterus into the medium, but no release from ileum or spleen could be detected

Discussion

Addition of antigen to sensitized rat tissues in the presence of an indicator segment of guinea pig ileum invariably produced contraction of the latter In terms of tissue weight this contraction was greatest for uterus moderate for spleen, and smallest for ileum A similar quantitative difference between guinea pig uterus and ileum was found by Boréus (1961)

When the indicator segment of guinea pig ileum was replaced with a segment of rat uterus which was about 1 000 times as sensitive to 5 HT no contraction of the indicator was observed. It was apparent therefore that contraction of guinea pig ileum had not been dependent upon released 5 HT

No anaphylactic release of 5 HT from guinea pig and rat tissues could be demonstrated even when ten times the quantity of tissue substrate was used and spectrophotofluorimetric assay carried out

Release' or no release of a substance are of course purely quantitative terms and dependent on the sensitivity of the method of identification The sensitivity of the rat uterus preparation was very high and our negative results signify that not more than 1 ng could have been released during the period of antigen contact This release is equivalent to less than 0 05 per cent and 0 04 per cent respectively of the total 5 HT content of the rat ileum and spleen The corresponding value for guinea pig ileum and spleen is less than 0 03 per cent

Consistent with these negative findings as to anaphylactic 5 HT release is the fact that the 5 HT content of antigen incubated ileum and spleen was not significantly lower than that of controls from the same animals Contrasting results emerged for rat uterus however Here the 5 HT level decreased 53 and 60 per cent after antigen incubation. It is difficult to evaluate the significance of this decrease since the 5 HT content of uterus is extremely low and the percentual values for small absolute differences are accordingly high In the guinea pig it was impossible to demonstrate any 5-HT even when 15 uteri were pooled and extracted A low 5 HT content has likewise been found in human uterus (Boréus Sandberg and Westerholm unpublished)

It seems possible that in rat uterus the decrease of the 5 HT level during antigen contact represented an anaphylactic release which might have contributed to the Schultz Dale contraction Since rat uterus is highly sensitive

to 5 HT, small amounts of the amine, liberated within the tissue, would suffice to stimulate the smooth muscle cells to contraction

In guinea pig tissues, however, nothing suggestive of 5 HT release during anaphylaxis was noted In this species the ileum and uterus are not very sensitive to 5 HT, and relatively large amounts would be required for anaphylactic contraction Under these circumstances a release would probably have been detectable in the experiments

To the question whether or not 5 HT is involved in the Schultz Dale reaction, there are two main experimental approaches An attempt to antagonize the contraction either by means of 5 HT inhibitors or by means of tissue desensitization with high doses of 5 HT before addition of antigen is one approach The other is the one used here — an attempt to demonstrate directly the release of 5 HT during the contraction

Conclusion as to the implication of certain liberated substances cannot readily be drawn from pharmacologic suppression of the Schultz Dale contraction since the specificity of the inhibitors are always in question Furthermore different segments of the same tissue vary considerably in their sensitivity to antigen (KEMPFF and FEINBERG 1948)

Evidence obtained with the method used here would be more reliable As far as we are aware 5 HT liberation during anaphylactic reaction *in vitro* has been observed only in the uterus and mast cells of the rat (GARCIA AROCHA 1961) As respects guinea pig, on the other hand, FINK and GARDNER (1958) stated that it was impossible to demonstrate release of 5 HT from sensitized lung, uterus or ileum in the presence of the highly serotonin sensitive mouse uterus The degree of 5 HT sensitivity of their preparation was not, however, stated SANYAL and WEST (1958) reported that a smooth muscle stimulating substance was obtained from pieces of spleen of sensitized guinea pigs, but no quantitative data on this point were given

That antigen *in vitro* releases much more histamine from guinea pig uterus than from ileum was shown by SCHILD (1939) Anaphylactic formation of a slow reacting substance is also greater in guinea pig uterus than in ileum (BORÉUS and CHAKRAVARTY 1960) It is interesting to note that a similar quantitative difference in antigen induced release of histamine is also found in rat tissue

Our results do not support the view that 5 HT is released during the Schultz Dale reaction in the guinea pig However, 5 HT may be partly responsible for the anaphylactic contraction of the rat uterus

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Release of 5-Hydroxytryptamine and Histamine from Rat Mast Cells[1]

By

N C MORAN[2], B UVNÄS and BARBRO WESTERHOLM

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Abstract

MORAN N C B UVNÄS and B WESTERHOLM *Release of 5-hydroxytryptamine and histamine from rat mast cells* Acta physiol scand 1962 *56* 26—41 — The release of 5 hydroxytryptamine (5 HT) and histamine from rat peritoneal mast cells was studied Major variations of amine content per cell were noted Compound 48/80 n-decylamine polymyxin B phospholipase A and distilled water released 5-HT and histamine The concentration response curve for 5-HT to 48/80 was parallel to that of histamine The number of histamine molecules released considered as a ratio of the number of 48/80 molecules in the medium ranged from 4 to over 40 whereas the corresponding ratio for 5-HT ranged from less than 1 to approximately 2 The amine release by 48/80 was dependent upon pH and temperature and took place within 20 sec at 22 C Certain enzyme inhibitors such as allicin (SH group inhibitor) and ninhydrin (NH_2 group inhibitor) blocked the amine release Dinitrophenol inhibited the release only in glucose free medium Sodium cyanide 10^{-4}M and anoxia inhibited the release of neither amine The results suggest that 5-HT is released from mast cells by the same mechanism as is histamine The data are in agreement with previous evidence from this laboratory favouring an enzymatic mechanism of release of histamine by polymer amine releasing agents

The presence of 5-hydroxytryptamine (5-HT) in isolated mast cells of the normal rat (BENDITT *et al* 1955 KELLER 1957 GARCIA AROCHA 1961a) and in the cells of mouse mastocytoma (FURTH HAGEN and HIRSH 1957, SJOERDSMA

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WAALKES and WEISSBACH 1957 GIARMAN POTTER and DAY 1960 GREEN and DAY 1960) is now well established although available evidence suggests that mast cells of other mammalian species do not contain this amine (SJOERDSMA *et al* 1957 PARRATT and WEST 1957a WEST 1957) PARRATT and WEST (1957b 1958) contend on the basis of ratios of 5 HT to tissue mast cells and of demonstrations of selective release of tissue histamine and 5 HT that even in the rat there is no uniform evidence that the 5 HT in rat skin is in mast cells

Evidence is also available showing a release of 5 HT in response to chemical histamine liberators from mast cells of rats (BHATTACHARYA and LEWIS 1956 GARCIA AROCHA 1961a) and from mouse mastocytoma cells in response to alkaline tissue extract (GIARMAN *et al* 1960) GARCIA AROCHA (1961b) has also demonstrated an antigen induced release of both histamine and 5 HT from peritoneal mast cells removed from sensitized rats

It is not certain whether the two amines are released by the same mechanism WEST (1959) in a review of tissue amines and mast cells cites a number of studies the results of which suggest that 5 HT is not released from the tissues of most species by histamine liberators GARCIA AROCHA found release of only 5 HT when antigen was added to minced uterus from sensitized rats and also an earlier release of 5 HT than of histamine from the perfused hind limbs of sensitized rats (1961b)

Previous work from this laboratory has produced evidence supporting the concept that degranulation of mesentery mast cells *in situ* and histamine release from isolated rat peritoneal mast cells by antigen and by polymer amine releasing agents is dependent upon enzymatic processes (HOGBERG and UVNAS 1960 UVNAS and THON 1960 1961) The present experiments were designed to determine if 5 HT is released simultaneously with histamine from rat mast cells and whether the mechanisms are similar Compound 48/80 was used as a model releasing agent because of its known characteristics as a histamine liberator and because the pattern of release of histamine by 48/80 is similar to that by antigen (HOGBERG and UVNAS 1960 MOTA and ISHII 1960)

Methods

Preparation of cells

Mixed peritoneal cells were obtained from male albino rats (Wistar strain) weighing 200 to 450 g The animals were anaesthetized with ether and exsanguinated by cutting the neck The abdominal muscle wall was exposed and a small opening was made by thermocautery through which was poured 7 ml of a buffered solution (pH 6 8—7) containing NaCl 1 54 $\times 10^{-1}$M KCl 2 7 $\times 10^{-3}$M anhydrous $CaCl_2$ 9 $\times 10^{-4}$M 10 percent Sorensen phosphate buffer (Na_2HPO_4 2 H_2O 3 $\times 10^{-3}$M KH_2PO_4 3 5 $\times 10^{-3}$M) dextrose 5 6 $\times 10^{-3}$M and albumin 1 mg per ml After closing the opening the abdomen was gently massaged for 90 sec and then widely opened in the midline by thermocautery The fluid was gently removed with a pipette and

centrifuged at 175 g for 3 min. The supernatant was discarded and the cells at the bottom of the tube were resuspended in the buffered solution mentioned above (2 ml of solution per rat) In most experiments cells from two or more rats were pooled

Isolated mast cells were prepared from the peritoneal cell suspension according to the Ficoll density gradient method described by UVNÄS and THON (1960)

Incubation Technique

Two ml of the cell suspension were poured into 25 ml beakers which were slowly rocked in a Warburg apparatus at 37 C After 10 to 15 min to allow temperature equilibration a releasing agent was added Following incubation with releaser for 5 to 15 min the cell suspension was centrifuged for 3 min at 400 g To the supernatant was added 0 1 ml of 2 N HCl and to the cell fraction 1 2 ml of 0 1 N HCl Both samples were kept frozen until assay

When inhibitors were investigated the cells were preincubated with inhibitor for 15 min before the releasing agent was added

More detailed methods for certain types of experiments are described in the results

Mast Cell Counts

Mast cell counts were made by the method described by BRAY and VAN ARSDEL (1961)

Histamine and 5-HT Assay

Histamine was tested on the atropinized (atropine sulphate $1\ 5 \times 10^{-4}$ M) guinea pig ileum and its specificity demonstrated by blockade with mepyramine (5×10^{-7} M)

5 HT was determined spectrophotofluorimetrically by the method of BOGDANSKI *et al* (1956) 5-HT was shown to be stable at 37 C for over 30 min in the buffered medium alone and in the cells Also heating a solution of 5 HT for 10 min at 45° C caused no detectable loss of the amine

All histamine and 5-HT values are expressed as the free base

Materials

Compound 48/80 prepared as described by BALTZLY *et al* (1949) and phospholipase A obtained from bee venom were kindly supplied by Dr B Högberg AB Leo Hälsingborg Sweden

Allicin was prepared from garlic according to CAVALLITO and BAILEY (1944)

Human serum albumin was kindly supplied by Dr N Hellström of AB Kabi Strängnäs Sweden

Reserpine phosphate was generously supplied by Dr Albert J Plummer CIBA Pharmaceutical Products Inc Summit N J USA

Other substances such as n-decylamine trypsin cyanide dinitrophenol and ninhydrin were obtained from commercial sources

Results

Content of 5-hydroxytryptamine and histamine in suspensions of mixed peritoneal cells and isolated mast cells

Ninety percent or more of the cells removed from Ficoll were mast cells as judged by their staining reaction with toluidine blue The amount of amines recovered from the isolated cells accounted for 30 to 60 percent of

Table I Content of 5 HT and histamine in rat peritoneal cell suspension and in rat mast cells

	5 HT µg/rat n = 82	5-HT µg/10⁶ mast cells n = 37	Hist. µg/rat n = 82	Hist. µg/10⁶ mast cells n = 37	Ratio Hist 5-HT n = 82
Mean ± standard error	1.09 ± 0.17	1.34 ± 0.20	27.3 ± 1.2	31.5 ± 1.9	47.4 ± 3.4
Range	0.05—8.88	0.22—5.70	3.5—46.8	12.2—58.9	3.3—103

the content of amines in the original suspension of mixed cells This low percentage is attributed to the sedimentation into Ficoll of the larger mast cells only although the presence of some 5-HT and histamine in other types of cells in the original suspension cannot be discounted Other cells in the peritoneal cell mixture were primarily leukocytes with a few erythrocytes and unidentified cells

Major variations were encountered in the amount of 5-HT and histamine both in mixed peritoneal cells and in isolated mast cells Table I shows the variability in amine content per rat and per million mast cells in suspensions of mixed rat peritoneal cells There was also a great variation in the ratio of histamine to 5-HT in different rats It was assumed as an approximation, that all 5 HT and histamine extracted from the mixed cell suspensions was localized in the mast cells since mast-cell free peritoneal cell suspensions from rats contain no detectable 5 HT or histamine (MORAN and WESTERHOLM 1962) There appeared to be no obvious correlation between the content of amines per rat and the weight of the animals the number of cells or the season of the year

Time course of 48/80 induced release of 5-HT and histamine from peritoneal cells

The release of both 5-HT and histamine from mixed peritoneal cells took place in a very short period of time Fig 1 shows an experiment in which the percentage of amines released in response to 48/80 after only 4 min exposure to the releasing agent was equal to that resulting from periods of exposure of up to 34 min The spontaneous release at the end of the period was 15

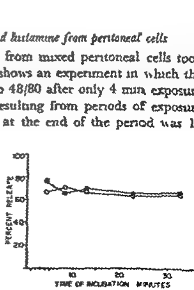

Fig 1 Time course of 5-HT and histamine release from mixed rat peritoneal cells ○ — 5-HT release by 48/80 0.5 µg/ml ● — Histamine release by 48/80 0.5 µg/ml

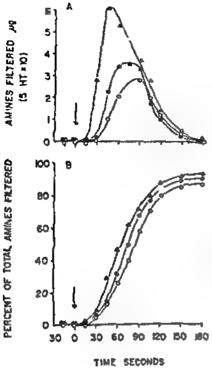

Fig. 2. Time course of 5-HT and histamine release from perfused isolated rat peritoneal cells. At arrow 40 μg of 48/80 was rapidly injected into funnel producing an immediate estimated concentration of 10 μg/ml. Filtrate collected in 15-second samples. *A* ○ — release curve for 5-HT; ● — Release curve for histamine; Δ — "washout" curve without cells; at arrow 4 μg of reference 5-HT injected instead of 48/80. *B* Reference 5-HT curve and release curves for 5-HT and histamine as a function of time. In this experiment 78 percent of the reference 5-HT was recovered and 93 and 97 percent respectively of the cellular 5-HT and histamine was released.

percent. In order to determine more precisely the time required for release of the amines, peritoneal cells were placed in a funnel lined with two layers of 00 Whatman filter paper. Standard incubation medium was perfused over the cells at room temperature (22° C) at a constant rate. The filtrate (free of cells) was collected in fifteen second increments and analysed for amines. By this method the time course of release of amines following addition of 48/80 to the perfusion medium could be determined. Two methods of administering 48/80 were used. In the first type, designed to give a brief exposure to a high concentration of the releaser, a single rapid injection of 48/80 was made into the funnel in an amount calculated to give immediate concentration of 10 μg/ml. In the second type, designed to give a continuous exposure to the releaser, a similar rapid injection of 48/80 was immediately followed by continuous perfusion with a new solution containing 48/80, 10 μg/ml. Samples of filtrate were collected for 150 to 180 sec. After collection of the final filtrate the filter paper was extracted with 0.1 N HCl to provide an estimate of the total amines in the cell suspension as a basis for calculating the percent released. Fig. 2 A illustrates the release curves for 5-HT and histamine in one experiment in which a single injection of 48/80 was given. The amines appeared in the filtrate within 30 sec after addition of the releaser and reached a peak by 75 to 90 sec. To compare these curves with the simple "washout"

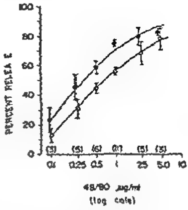

Fig 3 Concentration — response curves of release of 5 HT and histamine in response to 48/80 Each point represents the mean percentual release of 5-HT — ○ — and histamine — ● — Vertical bars represent standard errors figures in parentheses the number of experiments at each concentration. All values corrected for spontaneous release The results of experiments with both mixed peritoneal cells and isolated mast cells were pooled in order to obtain the means

of an amine incubation medium was passed through filter paper without cells and the content of 5 HT measured in the filtrate following the rapid injection of a reference amount of 5 HT into the funnel Comparisons were made between filtration of reference 5 HT and released amines only when the flow rates in the two instances were comparable Fig 2 A shows that the appearance and the peak of reference 5 HT are only slightly earlier than those of the released amines This is illustrated more clearly in Fig 2 B in which the percent of the total amines filtered is plotted as a function of time It can be seen that the curves for the released amines are parallel to that of the reference 5 HT with a lag of only 15 to 20 sec. Other experiments showed the same short lag period Thus the entire release of the amines in response to a high concentration of 48/80 (producing greater than 90 percent release) took place within a period of less than 20 sec at room temperature a graphic demonstration of the explosive release of histamine and 5 HT from mast cells Preliminary experiments have shown that n-decylamine causes an equally rapid release

Influence of dose of 48/80 upon the release of 5-HT and histamine

The magnitude of the release of both 5 HT and histamine from mixed peritoneal cells and isolated mast cells is dependent upon the concentration of 48/80 Fig 3 summarizes the dose response relationships of both mixed and isolated cells Each point represents the mean percentual release of the amine as a function of the dose of 48/80 The curves are parallel but the percentual release of 5 HT is slightly less than that of histamine However in some individual experiments there was no difference The difference is significant by paired *t* test at only two doses (0 5 µg/ml $p < 0.05$ 1 µg/ml $p < 0.001$)

The ratio of the number of molecules of amine released from the cells to the number of molecules of 48/80 (expressed as base units) in the medium

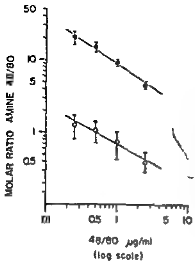

Fig 4 5 HT 48/80 and histamine 48/80 molar ratios as a function of the 48/80 concentration Each point represents the mean ratio of molecules of amine released to molecules of 48/80 (assumed molecular weight 170) in four experiments Vertical bars — standard errors ○ — 5 HT ● — histamine Values corrected for spontaneous release

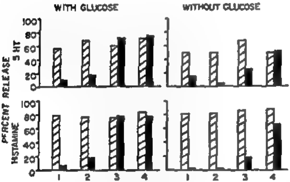

Fig 5 Effect of inhibitors on 48/80-induced release of 5-HT and histamine from rat peritoneal cells with and without glucose in the incubation medium Cross-hatched columns indicate release with 48/80 alone (1 μg/ml) black columns release with 48/80 in presence of inhibitor 1 Allicin 10^{-4} M 2 Ninhydrin 10^{-4} M 3 Dinitrophenol 10^{-} M 4 Sodium cyanide 10^{-} M Values corrected for spontaneous release

decreased with increasing concentrations of 48/80 The molar ratio of histamine to 48/80 varied from 2 8 to 47 in four experiments, the former at a concentration of 5 μg/ml and the latter at a concentration of 0 1 μg/ml of 5 HT In no instance was a 1 1 ratio observed In contrast, the ratio 5-HT 48/80 ranged from 2 14 to 0 13 Fig 4 shows the mean molar ratios in selected experiments at concentrations of 48/80 from 0 25 to 2 5 μg/ml When the ratios are plotted as a function of the concentration of 48/80 in the medium parallel curves are obtained

Influence of pH upon release of amines

Release of both 5-HT and histamine from peritoneal cells in response to 1 μg/ml of 48/80 was maximal from pH 6 8 to 8 2 but declined sharply with decreasing pH For example in one experiment there was less than 20 percent release at pH 5 4 approximately 50 percent release at pH 6 3, and between 60 and 70 percent at pH 6 8 7 5 and 8 1

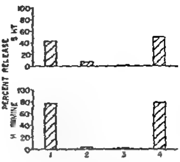

Fig 6 Release of 5 HT and histamine from isolated mast cells 1 48/80 1 μg/ml 2 48/80 1 μg/ml after preincubation with allicin 10^{-4} M 3 48/80 1 μg/ml after preheating of the cells for 10 min at 47 C 4 Phospholipase A 10 μg/ml Values corrected for spontaneous release

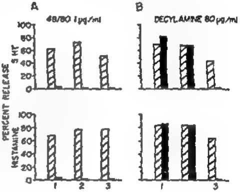

Fig 7 Comparison of the releasing actions of 48/80 and n decylamine on peritoneal cells A 48/80 1 μg/ml B n Decylamine 80 μg/ml Cross-hatched columns release in response to liberator alone Solid bars release after inhibitory procedure 1 Response in presence of allicin 10^{-4} M 2 Response after preheating of cells for 10 min at 47 C 3 Response at 0 5 C (In 3 the cross hatched bars represent control cells which had been cooled to 0 5 C for 30 min and then rewarmed to 37 before the addition of 48/80)

Effect of other agents upon release of amines

n Decylamine induced release of 67 to 71 percent of 5 HT and 84 to 89 percent of histamine from mixed peritoneal cells at a concentration of 80 μg/ml but caused no release at concentrations of 10 20 and 40 μg/ml Polymyxin B in one experiment released both 5 HT and histamine in a dose dependent manner 1 μg/ml releasing 25 and 15 percent of 5 HT and histamine respectively, 10 μg/ml, 57 and 57 percent and 100 μg/ml 80 and 85 percent Distilled water (mixed cells of one rat in 2 ml of water) released 95 and 96 percent respectively of 5 HT and histamine Incubation of mixed cells for 5 hours in lyophilyzed reserpine phosphate in concentrations up to 150 μg/ml caused release of neither amine

Hogberg and Uvnas (1957 1960) have suggested that the release of histamine from mast cells by compound 48/80 is triggered by the activation of a lytic enzyme attached to the mast cell membrane Of a large number of enzymes investigated only phospholipase A was found to cause degranulation of rat mesentery mast cells We tested two enzymes for their ability to release 5 HT and histamine Trypsin at a concentration of 1 000 μg/ml had no releasing action on mixed peritoneal cells Phospholipase A was capable of releasing both amines from isolated mast cells (Fig 6) as well as from mixed cells

Effect of chemical inhibition upon release of amines

Selected chemical inhibitors were tested Allicin a sulfhydryl inhibitor prepared from garlic and shown by HÖGBERG and UVNAS (1958) to antagonize the 48/80-induced degranulation of rat mesentery mast cells inhibited the release of both 5 HT and histamine in 48/80 treated peritoneal cells (Fig 5) and from isolated mast cells (Fig 6) The inhibition was nearly complete at 10^{-4} M concentration was less than 25 percent at 10^{-4} M and was absent at 10^{-4} M Allicin did not inhibit the release of amines evoked by n decylamine (Fig 7)

Ninhydrin thought to block NH_2 groups was similar to allicin in inhibiting the release of both 5 HT and histamine from mixed peritoneal cells 10^{-4} M concentration producing nearly complete antagonism (Fig 5) with little inhibitory action at lower concentrations

Dinitrophenol inhibited the 48/80 induced release of amines at a concentration of 10^{-4} M only if dextrose was not in the medium, whereas sodium cyanide at the same concentration had no blocking effect (Fig 5)

None of the inhibitors alone in concentrations of 10^{-4} M caused release of amines

Influence of temperature upon release of amines

UVNAS and THON (1961) demonstrated that 48/80 induced release of histamine from isolated rat mast cells was reversibly inhibited by low temperatures and irreversibly inhibited by temperatures above 44 C In our experiments the release of both 5-HT and histamine brought about by 48/80 was completely antagonized at a temperature of 0 5 C but was restored by rewarming the cells to 37 (Fig 7) In these experiments the cell suspensions were incubated in an ice bath in which the temperature was maintained at 0 5° C To one set of flasks 48/80 was added and after 15 min the suspensions were transferred to chilled tubes and rapidly centrifuged to separate cells and supernatant. The other set of flasks was then rewarmed to 37 C before addition of releasing agent The effect of n-decylamine was also reversibly inhibited at this low temperature (Fig 7)

At the other extreme heating the cell suspension for 10 min at 47 5 C a procedure which in itself caused no release of amines, prevented the 48 80 induced release of both 5-HT and histamine from mixed peritoneal cells (Fig 7) and from isolated mast cells (Fig 6) However, the action of n-decylamine was not influenced by this treatment (Fig 7)

Release of amines under anoxic conditions

Although it has been observed that anoxia inhibits the 48/80-induced degranulation of rat mesentery mast cells as well as the release of histamine from lung tissue (DIAMANT and UVNAS 1961) from rat diaphragm (ROTHSCHILD, VUGMAN and ROCHA e SILVA 1961) and from cat skin (WESTERHOLM

1960) we were unable to demonstrate an inhibition of release of either 5 HT or histamine from mixed peritoneal cells by incubation in an oxygen free atmosphere For these experiments the solutions were prepared from freshly boiled water The cell suspensions were placed in Warburg flasks with 48/80 in the side arms Nitrogen (or argon) was liberally flushed through the flasks then bubbled through the suspensions and finally the flasks were closed while a continuous stream of the gas was passing through After 45 min of pre incubation at 37 C 48/80 was added from the side arms without opening the flasks After a subsequent period of 15 min the flasks were cooled to 3 to 4 C in an ice bath before opening The contents were then quickly transferred to chilled tubes and rapidly centrifuged Since cooling had been shown to inhibit the 48/80-induced release it was felt that exposure to oxygen during the brief period required for centrifugation would have no effect on the chilled cells Release of amines resulting from the addition of 10 μg/ml of 48/80 was as great in cells incubated in nitrogen (99 7 percent pure) or in argon (5 parts of oxygen per million) as in air One experiment was performed in which a mixed cell suspension was incubated at 37 C for 3 hours in a dextrose free medium before replacing the air with nitrogen in order to exhaust possible energy yielding substances in the cells The release of both amines was comparable to that in a control suspension (without dextrose) under air These findings confirm the results of UVNAS and THON (1961)

Discussion

The present demonstration of 5 HT in the mast cells of the rat confirms the work of others (see introduction for references)

The amounts of amines in rat mast cells agree on the whole with those reported by other workers For instance GARCIA AROCHA (1961a) found 19 9 μg of histamine and 0 59 μg of 5 HT per rat in the peritoneal washings KELLER (1957) reported values of histamine from 23 to 40 μg per million rat mast cells and an average of 0 4 μg of 5 HT per million cells From the data of BENDITT *et al* (1955) one can calculate about 12 6 μg of histamine and about 0 8 μg of 5 HT per million rat mast cells Similar figures for histamine have been recorded (ARCHER 1958 BOREUS and CHAKRAVARTY 1960 LAGUNOFF and BENDITT 1960 BRAY and VAN ARSDEL 1961) Table I summarizes the values from a number of our experiments

The variation in the amount of amines and in the ratios of histamine to 5 HT (Table I) cannot be adequately explained PARRATT and WEST (1957a) have reported substantial changes in the concentration of histamine and 5 HT in the skin of rats — changes which are related to birth weaning lactation, and thyroxine feeding There was no corresponding increase in mast-cells It is possible that the wide variations we observed could be similarly ex

The precise localization of histamine and 5-HT is believed to be in the granules of mast cells. HAGEN, BARRNETT and LEE (1959) found histamine, 5-HT and heparin in specific granules of mouse mastocytoma cells. These granules were clearly separable from mitochondria and other intracellular organelles. To our knowledge 5-HT has not yet been localized to the granules of rat mast cells.

That polymer amines, such as 48/80, are capable of releasing 5-HT as well as histamine now seems certain. BHATTACHARYA and LEWIS (1956) demonstrated release of both amines from the perfused hind quarters of the rat. GARCIA AROCHA (1961a) has reported the release of 5-HT from rat peritoneal cells in response to 48/80 with results generally comparable to ours (see below) as well as by 1, 10-diaminodecane. GARCIA AROCHA (1961b) also noted an antigen induced release of 5-HT from mast cells obtained from sensitized rats. GIARMAN *et al.* (1960) reported concomitant release of 5-HT and histamine from suspensions of mouse mastocytoma cells in response to alkaline tissue extracts.

The mechanisms underlying the release of 5-HT and histamine from rat mast cells are apparently the same. The nearly identical time course of release of the two amines caused by 48/80, the similar dose response relationships with 48/80, the lack of selective release of either amine alone by any of the releasing agents tested, the comparable inhibition of release by chemical and thermal means and the similar pH dependence all point to this conclusion. The higher percentual release of histamine than of 5-HT would imply a difference in mechanisms were it not for the parallel dose response curves. GARCIA AROCHA (1961a) observed a similar discrepancy. No satisfactory explanation of this difference is yet available.

Nor is it necessary to postulate divergent mechanisms on the basis of the higher amine : 48/80 molar ratio for histamine than for 5-HT. The parallelism of the two molar ratio curves as a function of the concentration of 48/80 is indicative of a common mechanism. The simplest explanation of this difference lies in the disparate amounts of the two amines in the mast cells provided one assumes that the site of action of the liberators (for example the cell membrane) is remote from the storage sites of the amines (*e.g.*, the granules). Thus, if a liberator acts by altering the properties of the cell membrane so that the granules disappear from the cell, or by changing the intracellular milieu so as to promote diffusion of the amines from the granules and cells, the amount of amines which the cell loses will be proportionate to the amount originally bound in the cell. In order to account for the concentration-dependent release of amines by 48/80 on this basis, it seems necessary to postulate a quantical or all or none response, *i.e.*, a certain fraction of the cell population will be sensitive to a given dose of 48/80. No evidence to support these conjectures is yet forthcoming. Indeed, it is not known whether

5 HT and histamine are contained within the same granules of a cell or even whether they are contained in the same cells

A current hypothesis regarding the mechanism of release of histamine in response to polymer amine liberators and antigen is that of HOGBERG and UVNAS (1957 1958 1960) They have postulated activation of a lytic enzyme on the cell surface triggering in turn an energy requiring release mechanism which would result in liberation of intracellular amines Our results pertaining to the influence of temperature pH and inhibitors as well as to the effects of enzymes on the release of the amines are in accord with this hypothesis

Our inability to inhibit 48/80-induced release of amines from rat mast cells by anoxia conflicts with the findings of certain investigators who have used other preparations (DIAMANT and UVNAS 1961 rat lung WESTERHOLM 1960 cat skin ROTHSCHILD VUGMAN and ROCHA E SILVA 1961 rat diaphragm) and who have found anoxic inhibition of histamine release in glucose free medium Our findings however accord with those of UVNAS and THON (1961) who noted that anoxia did not antagonize the release of histamine from isolated rat mast cells These discrepancies the causes of which are not clear bring to mind the reported dependence of cellular enzymes on intact SH groups For instance according to RAPOPORT and SCHEUCH (1960) a pyrophosphatase in reticulocytes easily loses its activity by oxidation of essential SH groups unless the latter are protected by the presence of reduced glutathione (GSH) GSH in turn is conserved by the presence of glucose or glucose 6 phosphate due to the activity of hexokinase glucose 6 phosphate dehydrogenase and glutathione reductase SH groups appear to be essential for the release of amines from isolated mast cells since the release is blocked by SH blocking agents such as allicin and the inhibitory effect of allicin is antagonized by glutathione (UVNAS and THON 1961) Furthermore the sensitivity of isolated mast cells to liberators which activate the enzyme dependent releasing mechanisms is retained only when the cells are isolated in the presence of albumin which contains SH groups (UVNAS and THON 1961) or of GSH (UVNAS and THON unpublished observations) The lack of dependence of isolated mast cells upon glucose could be due therefore to the fact that the cells under our experimental conditions are supplied with enough SH groups to satisfy the needs of the enzymes of the releasing mechanisms

The release whatever its mechanism may be occurs very rapidly from mast cells (our results) and from tissues (FELDBERG and PATON 1951 PATON 1956) and indeed has been termed explosive To judge from the limited observations on record however the release of histamine from isolated mast cell granules if it takes place at all is a slow process COPENHAVER NAGLER and GOTH (1953) and HAGEN (1954) using dog liver large granule preparations (particles which MOTA *et al* (1954) found to be mast cell granules because of their metachromatic staining properties) were unable to demonstrate any release of histamine *in vitro* by chemical liberators such as stilbamidin

The precise localization of histamine and 5 HT is believed to be in the granules of mast cells HAGEN, BARRNETT and LEE (1959) found histamine 5 HT and heparin in specific granules of mouse mastocytoma cells These granules were clearly separable from mitochondria and other intracellular organelles To our knowledge 5 HT has not yet been localized to the granules of rat mast cells

That polymer amines such as 48/80, are capable of releasing 5 HT as well as histamine now seems certain BHATTACHARYA and LEWIS (1956) demonstrated release of both amines from the perfused hind quarters of the rat GARCIA AROCHA (1961a) has reported the release of 5 HT from rat peritoneal cells in response to 48/80 with results generally comparable to ours (see below), as well as by 1, 10-diaminodecane GARCIA AROCHA (1961b) also noted an antigen induced release of 5 HT from mast cells obtained from sensitized rats GIARMAN *et al* (1960) reported concomitant release of 5 HT and histamine from suspensions of mouse mastocytoma cells in response to alkaline tissue extracts

The mechanisms underlying the release of 5 HT and histamine from rat mast cells are apparently the same The nearly identical time course of release of the two amines caused by 48/80, the similar dose response relationships with 48/80 the lack of selective release of either amine alone by any of the releasing agents tested the comparable inhibition of release by chemical and thermal means and the similar pH dependence all point to this conclusion

The higher percentual release of histamine than of 5 HT would imply a difference in mechanisms were it not for the parallel dose response curves GARCIA AROCHA (1961a) observed a similar discrepancy No satisfactory explanation of this difference is yet available

Nor is it necessary to postulate divergent mechanisms on the basis of the higher amine 48/80 molar ratio for histamine than for 5 HT The parallelism of the two molar ratio curves as a function of the concentration of 48/80 is indicative of a common mechanism The simplest explanation of this difference lies in the disparate amounts of the two amines in the mast cells provided one assumes that the site of action of the liberators (for example the cell membrane) is remote from the storage sites of the amines (*e g* the granules) Thus if a liberator acts by altering the properties of the cell membrane so that the granules disappear from the cell or by changing the intracellular milieu so as to promote diffusion of the amines from the granules and cells the amount of amines which the cell loses will be proportionate to the amount originally bound in the cell In order to account for the concentration dependent release of amines by 48/80 on this basis, it seems necessary to postulate a quantical or all-or none response *i e* a certain fraction of the cell population will be sensitive to a given dose of 48/80 No evidence to support these conjectures is yet forthcoming Indeed it is not known whether

weight of the rats and to the amount of amines in the cells Since no figures were given a comparison with our observed variations is not possible He found as did we a consistently greater percentual release of histamine than of 5 HT GARCIA AROCHA states that the release of amines due to 48/80 and 1 10-diaminodecane (DA_{10}) was slow 60 percent of the histamine and 40 percent of the 5 HT being released in the first ten minutes This is in contrast to the extremely rapid release that we observed Furthermore his doses of 48/80 were 60 to 100 times higher than ours Although we cannot define the causes of this difference in rate of release and sensitivity of the cells it seems likely that his method of suspending the cells in isotonic sucrose may be the explanatory factor UVNAS and THON (1960) have found that cells isolated in sucrose do not retain their histamine throughout the isolation procedure and are less sensitive to the releasing action of 48/80 than are cells isolated in Ficoll Moreover UVNAS and THON (1960 1961) have shown that effective release can be obtained with low concentrations of 48/80 only if the cells are, throughout the isolation procedure and during incubation suspended in a medium containing either serum albumin or glutathione

Our inability to demonstrate reserpine induced release of 5 HT from mast cells *in vitro* could imply that reserpine does not enter these cells or that the 5 HT in the mast cells is bound in a different manner (or is contained in particles of different characteristics) than the 5 HT in the brain the blood platelets and in argentaffine cells of the small intestine It is also possible that the short duration of mast cell exposure to reserpine (five hours) is in adequate Experiments in which rats were pretreated with a large dose of reserpine 24 hours before removal of the mast cells showed that the amount of 5 HT per cell fell to 65 per cent and histamine to 82 per cent of the initial level (MORAN and WESTERHOLM 1962)

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Distribution of Red Cells and Plasma in Rabbit and Cat Kidneys

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Abstract

ULFENDAHL, H. R. *Distribution of red cells and plasma in rabbit and cat kidneys.* Acta physiol. scand. 1962. 56. 42—60. — The distribution of red cells and plasma in rabbit and cat kidneys were studied with regard to the cell separation theory — postulated by PAPPENHEIMER and KINTER. Methods for measuring volumes of red cells and plasma in organs or parts of organs have been critically examined. The use of Cr^{51} red cells and I^{131} albumin were found to be useful in measuring red cell and plasma volumes in sections of frozen kidneys. The red cell volume in cat kidneys was found to be 0.034 ml and the plasma volume 0.129 ml per g kidney. The ratio between the intrarenal and the systemic arterial hematocrits was 0.64 for the cat and 0.73 for the rabbit. The outer part of the cortex had a higher red cell concentration than the central part and the medulla displayed a hematocrit gradient with the highest value at the border to the cortex and the lowest value at the tip of the papilla. The results seem to be in accordance with the cell separation theory but it cannot be stated whether the separation is of such a magnitude as to be of importance as a regulatory mechanism of renal function. During water diuresis a considerable increase in the red cell and plasma volumes occurred in the papilla.

The hematocrit ratio of the blood in most organs is different from that of the arterial blood. In this the kidney is no exception, the difference being rather high. Thus GIBSON *et al.* (1946) found the hematocrit of the dog kidney to be only 0.35 of the arterial hematocrit. Similar investigations with varying techniques have later shown low hematocrit values in kidneys (ALLEN and REEVE 1953, LEWIS, GOODMAN and SCHUCK 1952).

In 1955 PAPPENHEIMER and KINTER briefly reported their interpretations of the cause of the low hematocrit in the kidney and a year later published full details of the investigations that they concluded indicated that the low hematocrit values in the kidney were expressions of an intrarenal separation of red cells and plasma in the interlobular arteries Thus the outer glomeruli received a cell rich blood and the inner glomeruli a cell poor blood They further assumed that the red cells had to pass through a short vascular path while the plasma had a longer pathway (KINTER and PAPPENHEIMER 1956 a and PAPPENHEIMER and KINTER 1956)

On the basis of their theory they tried to elucidate some physiological renal phenomena of which the mechanisms were unclear Thus they assumed that the separation theory could explain the autoregulation of renal blood flow and glomerular filtration the variations of the extraction ratios of Diodrast and PAH and the low oxygen tension in the urine (PAPPENHEIMER and KINTER 1956 KINTER and PAPPENHEIMER 1956 a and b)

Many authors have been inspired by the cell separation theory Most of them have studied the consequences of an intrarenal separation of red cells and plasma on the kidney function The results of most of these investigations did not confirm the occurrence of a separation

Only a few workers have attacked the problem by a more direct study of the actual separation

The aim of the present investigation was to determine whether the distribution of red cells and plasma in different regions of the kidney was consistent with the cell separation theory

The first part of the paper deals with the methodological problems which arise in the measurement of the intrarenal red cell and plasma volumes In the second part a detailed study of the distribution of red cells and plasma in the kidney is described The results are in favour of an intrarenal separation but do not give any information about its consequences on kidney function

PART I

Methods

Operative procedure Rabbits were anesthetized i v with Nembutal (ABBOT) 40 to 50 mg/kg The left kidney was exposed by a midline abdominal incision and freed from the surrounding fat Two loose ligatures were placed around the artery and the vein A plastic tube was tied into the left ureter and the urine secretion was measured with a graduated cylinder

A two per cent solution of a local anesthetic was injected into the tissue around the renal pedicle to block the nerves passing to the kidney

One of the carotid arteries was cannulated and the arterial pressure measured with a mercury manometer

Preparation of radioactive red cells and plasma At least 2 hours before the operation 5 to 10 ml blood was taken from a marginal vein of the rabbit ear into a heparinized

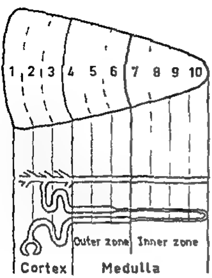

Fig 1 The diagram shows the cutting plan for the slices of rabbit kidney In cat kidney the cortex was cut into 6 slices

centrifuge tube and after centrifugation and removal of plasma 50 to 100 μC of Cr^{51} as sodium chromate were added After incubation for 1 to 2 hours the red cells were washed 5 times and 5 to 10 μC I^{131} human serum albumin were added Rabbit plasma was added to make the total volume up about 10 ml The radioactive albumin solution (Radiochemical Center Amersham England) was tested by paper electrophoresis (BILL MARSDEN and ULFENDAHL 1960) and found to contain more than 2 per cent The amount of radioactive albumin varied between 1 and 8 mg

Experimental procedure A high secretion rate of urine 0 1 to 1 5 ml/min was produced v injection of 0 5 g urea and 0 5 g glycine In order to decrease the accumulation free radioactive iodide in the kidney 3 ml of a 0 167 M sodium iodide solution was so injected i v as a carrier

30 sec or 3 min after the injection of the radioactive blood into a vein the kidney after rapid ligation and division of the pedicle was placed in a vessel containing isopentane cooled by liquid air Immediately afterwards an arterial blood sample from the left ventricle of the heart was taken in a heparinized syringe for the analyses of hematocrit and radioactivity

The kidneys were cut in a small chamber cooled with CO_2 ice into 2 mm thick slices These were freed from the remaining parts of the capsule and put into small glass tubes for weighing and radioactive analyses (Fig 1)

Determination of blood hematocrit Arterial blood was centrifuged at [illegible] 600 × *g* for 3 min (ENGHOFF 1937) No corrections for trapped plasma were made

Radioactive analyses and calculation of tissue hematocrit The analyses of Cr^{51} and I^{131} were performed simultaneously in a two channel gamma spectrometer with a well type NaI crystal using the method described by ÖBRINK and ULFENDAHL (1959) An automatic sample changer which printed the results was used (ÖBRINK and ULFENDAHL 1960 unpublished) in most of the experiments

The energy channels for counting Cr^{51} and I^{131} in the present study were different from those described by ÖBRINK and ULFENDAHL (1959) The channels selected are shown in Fig [illegible]

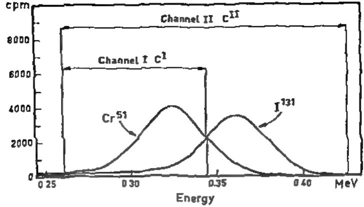

Fig 7 Gamma energy spectrum of Cr^{51} and I^{131} This choice of channels will give high stability and counting rates

In one channel (C^{II}) the window was quite open and the influence of a long time drift was reduced as far as possible The choice of channels was also advantageous in that it gave high counting rates

In neither channel were less than 10 000 counts recorded

The stability of the apparatus was controlled by counting standard samples of Cr^{51} and I^{131} at least every hour

For *the calculations of the activities of the two nuclides* the following equations were used

$$C_{Cr}^{I}/C_{Cr}^{II} = q_{Cr} \tag{1}$$

$$C_{I}^{I}/C_{I}^{II} = q_{I} \tag{2}$$

$$C_{x}^{I}/C_{x}^{II} = q \tag{3}$$

$$f_{Cr} = (q - q_{I})/(q_{Cr} - q_{I}) \tag{4}$$

$$C_{Cr_x}^{II} = f_{Cr} \cdot C_{x}^{II} \tag{5}$$

$$C_{I_x}^{II} = C_x^{II} - C_{Cr_x}^{II} \tag{6}$$

C_{Cr}^{I} and C_{I}^{I} = counts per min of the Cr^{51} and I^{131} standards respectively in channel I

C_{Cr}^{II} and C_{I}^{II} = counts per min of Cr^{51} and I^{131} standards respectively in channel II

C_x^{I} and C_x^{II} = counts per min of both Cr^{51} and I^{131} in arterial or kidney samples in channels I and II (When the half lives of both nuclides are long $C_x^{II} = N^{II}$ according to ÖBRINK and ULFENDAHL 1959)

f_{Cr} = the fraction of activity in channel II originating from Cr^{51}

For details of the derivation of the equations see ÖBRINK and ULFENDAHL (1959) and BILL, ÖBRINK and ULFENDAHL (1959)

The volume of the red cells in the arterial blood samples was obtained by means of the following equation

$$RCV_a = W_a \cdot Hct_a/[Hct_a \cdot SW_{RC} + (1 - Hct_a) \cdot SW_P] \tag{7}$$

W_a = weight of arterial sample g

Hct_a = hematocrit ratio of arterial blood

SW_{RC} = specific gravity of red cells (1 091)

SW_P = specific gravity of plasma (1 027)

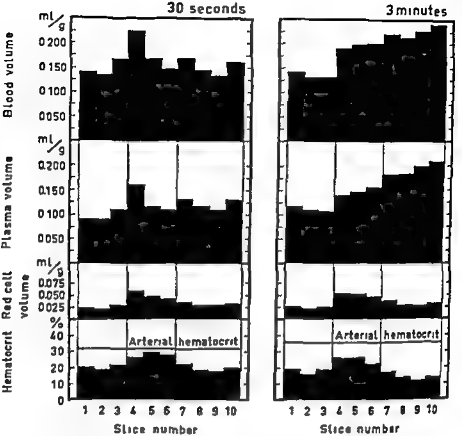

Fig 3 Average results from rabbit kidneys The left diagram shows the result from 5 kidneys removed after 30 seconds and the right from 8 kidneys removed after 3 minutes For slice number code see Fig 1

The volume of plasma in the arterial blood sample

$$PV = W \quad (1 - Hct_a)/[Hct_a \; SW_{RC} + (1 - Hct_a) \; SW_P] \tag{8}$$

The volumes of red cells and plasma in the kidney were calculated as follows

$$RCV_K = RCV \quad Cr_K^{II}/Cr_a^{II} \quad W_K \tag{9}$$

$$PV_K = PV \quad CI_K^{II}/CI_a^{II} \quad W_K \tag{10}$$

RCV_K = red cell volume of kidney tissue ml/g
PV_K = plasma volume of kidney tissue ml/g
W_K = weight of kidney sample g
Cr_K^{II} and CI_K^{II} = counts per min of Cr^{51} and I^{131}, in kidney samples in channel II

The error of the method was calculated from one sample counted 10 times in the spectrometer The error of a single determination was $\pm$ 2 5 per cent of the mean value

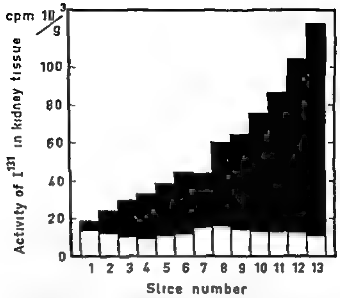

Fig 4 The accumulation of free radioactive iodide in a cat kidney during osmotic diuresis The black bars show the total radioactivity and the white the calculated radioactivity of blood in each slice. For slice number code see Fig 1 Slices 1—6 represent the cortex

Results

1 *The length of the mixing time* In order to study the influence of the length of the mixing time the rabbit experiments were performed in two series In the first the mixing time was 30 sec and in the second 3 min and it was apparent that there were no differences in the red cell content between the two series (Fig 3)

The arterial or venous blood in the pedicle of the frozen kidney taken after 30 sec had the same ratio of Cr^{51} to I^{131} as arterial blood taken 30—60 sec later thus indicated a satisfactory mixing of both the labelled red cells and plasma in the renal circulation

The calculated plasma volume was small in all parts of the kidney in the 30 seconds compared with the 3 minutes experiments Most accentuated was the difference in the papilla where the plasma volumes were 0 121 ml/g and 0 192 ml/g in the 30 seconds and 3 minutes experiments respectively The values for the cortex were found to be 0 095 and 0 110 ml/g and for the outer zone of the medulla 0 122 and 0 145 ml/g (Fig 3)

2 *Free iodide* Fig 4 shows the accumulation of radioactive iodide in different parts of a cat kidney tracer amounts of I^{131}-iodide were given i v 10 min before the removal of the kidney If carrier iodide was added the accumulation decreased The results of the investigation on the halogen distribution in the kidney are preliminary and will be described in detail in a later paper (Olsson Ulfendahl and Wallin)

3 *The size of the labelled particles in plasma* Attempts to use particles larger than radioactive albumin for labelling plasma were not successful, but will be described as they have some methodological value

a *Dextran labelled with sodium Cr^{51} chromate* A high molecular fraction of dextran was labelled with Cr^{51} chromate as according to JAKOBSSON and WIKSTROM (1958) No free Cr^{51} chromate was found after paper electrophoresis of the labelled dextran

The calculated plasma volumes with Cr^{51} dextran were always much higher than the plasma volumes simultaneously determined with I^{131}-albumin In the most favourable experiment the ratio between the two plasma volumes was 2

b *Plastic particles labelled with Br^{82}* JUHLIN (1956) used spherical plastic particles containing a fluorescent dye for a study of spreading in connective tissue In my experiments, the particles[1] were labelled instead with Br^{82} and were composed of 95 per cent methyl methacrylate and 5 per cent methyl bromine[81] acrylate With lauryl sulphate as emulsifier the diameter of the particles was 0 05—0 10 μ and with sorbitane monostearate (Span 60) 0 3—0 6 μ The particles were radiated in a nuclear reactor (AB Atomenergi Stockholm, Sweden) for not more than 24 hours with a neutron flux of 2×10^{11} n $\times$ cm^{-2} $\times$ sec^{-1} No changes in the particle suspension were detected in the dark field microscope after the radiation At higher neutron fluxes the particles always aggregated No radioactivity was found in the dialysate at dialysis

Before the experiments the animals were loaded with large doses of non radioactive plastic particles or Thorotrast (colloidal thorium dioxide)

The calculated plasma volumes were always larger than those found with I^{131} albumin

Method discussion

1 *Measurement of red cell volume* Cr^{51} chromate labelling of the red cells seems to be a satisfactory method since the high radioactivity facilitates a careful analysis of the red cell concentration in tissues

The amount of radioactive chromate that diffuses out of the cells must be negligible during the short duration of these experiments This is supported by the rabbit experiments where no differences of Cr^{51} activities were found between the 30 seconds and 3 minutes series (Fig 3 and Table I) The results also agree well with the values of PAPPENHEIMER and KINTER (1956) obtained by the chemical analysis of hemoglobin The experiments with Cr^{51}-dextran seem to show that free chromate will attach to the kidney tissue and the red cells must be carefully washed free from nonbound Cr^{51} chromate

EMERY *et al* (1959) in their investigation on dogs, used P^{32} as a red cell marker 1 to 3 per cent of the radioactive phosphate may leave the cells in half an hour, and if there is any tendency for phosphorus to accumulate in the kidney, the calculated red cell volume will be too high This danger may

[1] The plastic particles were kindly made by AB Nobelkrut Bofors Sweden

Table I The calculated red cell plasma and blood volumes and the ratio of renal hematocrit and arterial hematocrit of the total kidney

	Red cell volume ml/g	Plasma volume ml/g	Blood volume ml/g	Renal haematocrit / Arterial haematocrit
Cat osm. diuresis, 30 seconds	0 034	0 129	0 163	0 64
Cat antidiuresis 30 seconds	0 033	0 123	0 156	0 64
Cat, water diuresis 30 seconds	0 040	0 183	0.223	0 67
Rabbit, osm. diuresis, 3 minutes	0 034	0 126	0 159	0 59
Rabbit osm. diuresis 30 seconds	0 032	0 108	0.140	0 73

not in fact be great however because there are carrier amounts of phosphate in blood LILIENFIELD *et al* (1958 a) used Cr^{51} labelled red cells in distribution experiments on dogs but their results do not agree with those of EMERY *et al* Thus the values obtained by EMERY *et al* for the red cell volume in the cortex were about 20 per cent greater than those of LILIENFIELD *et al* In the medulla, however LILIENFIELD *et al* found the red cell concentration to be 25 per cent higher than did EMERY From the published results it is impossible to calculate the significance of the difference between the two investigations It is thus likely that the difference in the results depends on different labelling methods

2 *Measurement of the plasma volume* Several methods have been used for the measurement of the tissue plasma volumes The problem is to find a plasma labeller that does not leak through the capillary walls into the extravascular space or become attached to the walls of the vessels Thus the choice of the particle size is of importance but the electric charge and other physico chemical properties of the particles must not be neglected It may be possible that some part of the kidney tissue is able to take up large particles by a phagocytic mechanism (RHODIN 1958)

Even if the particles are unable to penetrate the glomerular membrane they may pass into the extravascular space elsewhere in the kidney Studies on renal lymph concentrations have shown the same quantitative protein pattern as in blood serum but the concentrations are only about half (BRIE and MAYERSON 1960) SWANN *et al* (1956) studied the albumin globulin ratio in blood plasma drained from the vein of ligated dog kidneys and found about the same ratio in the last part of the drained fluid as in arterial blood On the other hand LASSEN LONGLEY and LILIENFIELD (1958) found a higher concentration of I^{131} albumin in the papilla of dog kidney compared with I^{131} gamma globulin but only sparse quantitative data were given by the authors Summing up the results reported in the literature very few facts are known about the permeability of the peritubular vessels

The transfer of albumin through the vessel walls is further discussed below

a *Radioactive spherical plastic particles and high molecular dextran as plasma labellers* The author's attempt to use particles larger than the albumin molecules did not succeed The particle size varied between 0 6 and 0 05 μ However in spite of the magnitude of the particles great amounts of them appeared to have entered the extravascular space or to have become attached to the vessel walls It must be emphasized that during radiation in the reactor some of the Br^{82} could have been split off from the plastic particles and transferred to other molecules small enough to penetrate the membrane of the renal capillaries but not the cellophane membrane JUHLIN (1958) studied the distribution of spherical particles in certain organs and found a large uptake in liver and bone marrow and a small uptake in spleen and lung but no information was given concerning the accumulation in the kidney

To judge from the experiments with Cr^{51} dextran it seems quite clear that this labelling method cannot be used because the binding between Cr^{51} chromate and dextran breaks down intravascularly

b *The role of free I^{131} iodide* It must be stressed that even a low concentration of free I^{131} iodide in blood will yield a large amount of this ion in the kidney The accumulation ratio I^{131} iodide activity of kidney tissue to that of kidney blood will be 10 or more in the tip of the papilla but in the outer cortex only 2 or less (Fig 4) Even at a low concentration of free I^{131} iodide in plasma the accumulation in the kidney will result in an overestimate of the plasma volume and this is greatest in the papilla Carrier amounts of iodide will decrease the accumulation ratio Neither EMERY *et al* (1959) nor LILIENFIELD ROSE and LASSEN (1958) made any attempts to reduce the effect of the free I^{131} iodide in the determination of the plasma volume The latter however attempted to determine the content of non protein bound I^{131} in tissue homogenates and found it to be less than 2 per cent of the total I^{131} in the tissue But some of the tracer amounts of I^{131} iodide may have co-precipitated with the proteins How much this I^{131} iodide accumulation will increase the calculated plasma volume is impossible to say but it may be to a great extent, particularly in the medullary regions In both parts of this investigation the error of free I^{131} iodide was depressed by loading the animal with non radioactive iodide

c *The mixing time* The existence of a transcapillary transfer of albumin into the extravascular fluid of the kidney has been shown by the content of albumin in the renal lymph (BRIE and MAYERSON 1960) These experiments however did not give any information about the rate of this transcapillary exchange LILIENFIELD ROSE and PORFIDO (1957) found that the concentration of I^{131} albumin after 3 minutes in the dog papilla was 85 per cent of its concentration at one hour In my rabbit experiments the content of albumin in the papilla increased about 40 per cent when the mixing time was lengthened from 30 sec to 3 min In the rest of the kidney the increase was about 15 per cent

PAPPENHEIMER and KINTER found 0.183 ml plasma/g tissue of the total cat kidney, while the author's value is 0.129 ml/g for the cat (*cf.* part 2) and 0.108 ml/g for the rabbit. In the experiments of PAPPENHEIMER and KINTER it seems to be the longer mixing time combined with an accumulation of free I^{131} iodide that is responsible for the high plasma volume. Thus it is concluded that a short mixing time has considerable influence in the determination of the plasma volume in the papilla, while variation in the mixing time does not influence the red cell volume anywhere in the kidney.

d. *The method of removal of the kidney.* It is of great importance to prevent redistribution of the blood in the organ after stopping the circulation through it.

With the technique used some redistribution of blood may have taken place in the kidney from the time the pedicle was ligated until it was frozen in the cold isopentane.

The pressure difference existing between arteries and veins in the kidney is levelled immediately after ligation of the pedicle by the squeezing of blood from the arteries into the veins. Further, cold may induce an active vasoconstriction followed by a redistribution of blood. A third reason for a redistribution might be due to local volume variations caused by the temperature changes. The result may be a more diffuse pattern of the blood distribution compared with that of the intact kidney.

In the second part of the investigation the freezing technique was changed in order to fix the actual blood distribution to the greatest possible extent (*cf.* part 2). With this technique, where the kidney was frozen *in situ*, the vessels in the pedicle and also the superficial parts of the kidney were frozen immediately. It was assumed that no significant volume displacements occurred within the rigid mantle formed by the superficial frozen parts of the kidney.

PART II

Methods

Operative procedure. Cats were used as experimental animals and the narcosis was induced by ether and chloralose was administered i.v. (60—80 mg/kg in an 1 per cent solution).

Urine flow and arterial blood pressure were measured as in the rabbit experiments.

After dissecting free the pedicle a thin cotton thread was tied to the capsule and the kidney was hung up in a brass cylinder as shown in Fig. 5. The cylinder rested on a ring of foam plastic, avoiding compression of the blood vessels. To make the chamber so formed watertight a small amount of water glass was poured in the bottom of the cylinder.

The kidney was pharmacologically denervated by local anesthesia of the nerves in the pedicle.

Preparation of radioactive red cells and plasma. Immediately after anaesthesia blood was taken from the animal in order to label the red cells with Cr^{51} as in the rabbit experiments.

The content of free iodide was eliminated from the I^{131} human serum albumin as far as possible by a gel filtration method described by BILL *et al.* (1960).

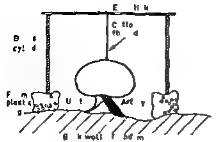

Fig 5 The arrangement for rapid freezing of cat kidneys

Experimental procedure An osmotic diuresis was induced by an injection of 0 5 g urea and 0 5 g glycine solved in 10 ml saline In 2 experiments injections of 0 45 per cent sodium chloride solution produced water diuresis In the experiments with antidiuresis Pitressin (Parke Davis & Co Ltd England) was given (0 5—1 0 pressor unit/kg body weight) All injections were given i v

About 30 sec after the i v injection of the labelled red cells and plasma a large volume of isopentane cooled by liquid air was poured over the kidney in the cylinder Arterial blood was taken from a carotid artery for the analyses of radioactivity and haematocrit ratio

The kidneys were cut up in the same way as the rabbit kidneys with the exception of the cortex which was cut into six parts All large vessels were cut away

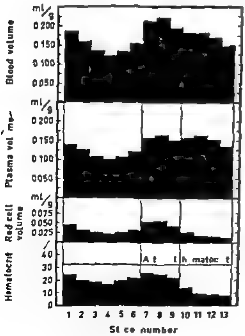

Fig 6

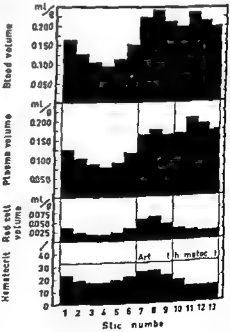

Fig 7

Fig 6 Average results from 9 cat kidneys during osmotic diuresis

Fig 7 Average results from 5 cat kidneys during antidiuresis induced by intravenous injection of Pitressin. For slice number code see Fig 1 Slices 1—6 represent the cortex

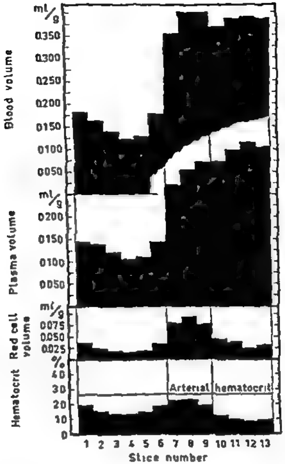

Fig 8 Average results from two cat kidneys during water diuresis For slice number code see Fig 1 Slices 1—6 represent the cortex

Results

In the cat experiments the mixing time was 30 sec The red cell and plasma distributions at different conditions of diuresis are shown in Fig 7 and 8

The character of the distribution curves of the red cells are nearly the same under the different conditions

The red cell volume is relatively large in the outer parts of the cortex, decreases in the middle part and increases again in the juxtamedullary region

In the outer zone of the medulla the red cell volume is as large as in the outer cortex but decreases progressively towards the tip of the papilla where the cell volume is only about one third of that in the outer zone

The variations of the plasma volumes in the cortex are conspicuously small between the experimental series but are larger in the medulla The

volume (and the red cell volume) seem to increase remarkably during water diuresis

The hematocrit pattern is very characteristic and is found to be similar in every experiment (Fig 6 7 and 8)

The differences in the hematocrit values between sections 1 and 2 ($P < 0\ 001$) and between 3 and 4 ($0\ 01 > P > 0\ 001$) are statistically significant, but not between 2 and 3 ($P > 0\ 1$) and 4 and 5 ($0\ 1 > P > 0\ 01$)

In order to obtain the red cell and plasma volumes representative of the total kidney the following calculations were made The mean values of the red cell and plasma volumes were calculated for the cortex the outer medulla and the inner medulla respectively From these and the mass ratios between the cortex (69 9 %) the outer medulla (22 8 %) and the inner medulla (7 3 %) given by VON MOLLENDORFF (1930) the red cell and plasma volumes of the total kidney were calculated. The results are shown in Table I

Discussion

The observed distributions of red cells and plasma and those predicted according to the cell separation theory

a *The separation of blood in a marginal plasma layer and an axial red cell core* It would be expected that the haematocrit ratio in the kidney would be much lower than that of the arterial blood because of the rich content of vessels with a diameter giving a thick marginal layer of plasma compared with the axial stream of red cells It is impossible to calculate exactly the magnitude of the volume of blood which streams in these vessels but an attempt at an approximate calculation is described below

According to WEAVER *et al* (1956) the circulatory system of the dog kidney can be filled with latex to a volume of 0 11 ml/g tissue These authors assumed the volume of the capillary system to be about 0 03 ml g giving for the whole dog kidney a blood content of 0 14 ml g as capillary vessels cannot generally be filled with latex If this value is compared with the blood volume obtained with the single injection technique it seems to be too small. LOCHNER (1954 found a value of 0 293 ml g LILLIENFIELD *et al* (1957) 0.24 ml/g and OCHWADT 1957) 0 20 ml g These investigations have perhaps given underestimated values (see the discussion below) but in spite of this the blood volume in the capillary vessels seems to be of about the same order of size as that of the large vessels WEAVER s value subtracted from LILIENFIELD s value will give 0 13 ml g)

WEAVER MCGARVER and SWANN (1956) did not find any latex filled vessels in the medulla an observation that is not in accord with the results of either this investigation or those of EMERY *et al* (1959) and LILIENFIELD *et al* (1958 a) Thus it seems clear that a large volume of the intrarenal

blood must be in small vessels where the axial flow is of great influence giving rise to a low intravascular hematocrit The ratio between the volume of large and small vessels varies in different regions of the kidney and this further complicates the intrarenal hematocrit pattern

The occurrence of large vessels in the transitional region between the cortex and the medulla leads here to a relatively high hematocrit

In the inner part of the cortex the wide proximal parts of the interlobular arteries and veins contain a great deal of blood with a relatively high hematocrit

In the outer zone of the medulla a higher hematocrit is to be expected than in the inner medulla because of a richer content of thicker vessels

The caliber of the vessel is not the only factor that affects the hematocrit the linear velocity of flow being also of great importance However nothing is known about the variations of this velocity in different regions of the kidney

b *Division of red cells and plasma into separate pathways* If there is a cell separation in the interlobular arteries the blood feeding the outer glomeruli should be cell rich and further the blood in the medulla should have a low hematocrit Our knowledge about the distribution of the blood after its passage through the glomeruli is very incomplete but on the whole the blood from the outer glomeruli must flow to the outer parts of the peritubular vascular system and the blood from the inner (juxtamedullary) glomeruli to the inner parts It is plausible that the hematocrit pattern existing in the interlobular arteries is partly blurred in the peritubular system

2 *Earlier experiments with similar techniques* Two groups of investigators used a similar technique for studying the distribution of red cells and plasma in the dog kidney (Lilienfield *et al* 1958a and Emery *et al* 1959) They only compared the hematocrit in the inner and the outer parts of the cortex and both found a higher hematocrit in the inner half. Lilienfield *et al* (1958 a) concluded that their results did not indicate a cell separation according to the theory of Pappenheimer and Kinter while Emery *et al* (1959) were more cautious and did not exclude intrarenal cell separation It should be mentioned however that the resolution of the hematocrit pattern in the cortex was too low and that measurement of the hematocrit in a greater number of serial cortical slices might have given a more correct picture

As regards the hematocrit patterns in the medulla the results of the two groups are remarkably different While Emery *et al* found the largest cell concentration in the papilla Lilienfield *et al* found in agreement with my results reversed conditions with the greatest concentration in the outermost part of the medulla. The reason for the difference in the results from the two groups is very likely the difference in techniques

3 *Determination of intrarenal hematocrit with the single injection technique* Lilienfield *et al* (1957 1958 b) and Ochwadt (1957) measured the blood flow and the mean transit times of red cells and plasma through dog kidneys

From the calculated red cell and plasma volumes they found the intrarenal hematocrit to be about 0 89 of the arterial hematocrit At first LILIENFIELD *et al* (1957) supported the cell separation theory but later (1958 b), as also did OCHWADT, they rejected it

The reasons for the different results obtained with the equilibration method and the single injection technique are problematical The equilibration techniques on dogs gave too low hematocrit values because of the accumulation of free radioactive iodide and extravascular I^{131} albumin The single injection technique may have given too high intrarenal hematocrits for the following reasons

a *Bad mixing in the renal artery* It must be difficult to obtain good mixing of the streaming blood in the renal artery and the injected labelled red cells and plasma The laminar blood flow may convey the injected blood to only a small area of the renal vascular tree and the calculated intrarenal red cell and plasma volumes will not therefore be representative for the whole kidney (WHITE 1939)

b *Uneven distribution of the plasma labeller in the cross section of the blood vessels* COPLEY and SCOTT BLAIR (1958) showed the existence of an immobile zone of plasma adjacent to the wall of artificial tubes and living blood vessels Graphic particles and Pontamen blue did not enter this immobile zone Thus the marginal plasma layer can be regarded as being divided into an outer immobile part and an inner region consisting of several mobile layers with higher velocities centripetally When there is no turbulence the particles have to diffuse perpendicularly to the long axis of the vessel in order to be distributed evenly With large particles the diffusion is slow and in the single injection technique an even distribution in the cross section of the vessel is never reached Thus the question of an uneven distribution cannot be neglected

It seems probable that both the equilibration and single injection techniques on dogs may have been liable to errors and that the ratio between renal and arterial hematocrits lies in reality between 0 49 (LILIENFIELD *et al* 1958a) and 0 89 (LILIENFIELD *et al* 1957) These values should be compared with the results in the present investigation 0 64 for the cat and 0 73 for the rabbit

4 *The site of the cell separation in the kidney* PAPPENHEIMER and KINTER postulated the separation of red cells and plasma in two regions of the renal vascular system

a in the interlobular arteries and
b in the peritubular vessels

a The separation in the interlobular arteries finds support in numerous experiments of PAPPENHEIMER and KINTER and in the investigation presented here It was also shown that the blood drained from the subcapsular veins of cat kidney has a higher red cell concentration compared with the arterial blood and that this difference is abolished at low arterial blood pressure (ULFENDAHL 1962)

b The assumption of the diversion of the postglomerular blood flow into two parallel circulations a short cell rich and a long cell poor route is more hypothetical The hypothesis seems to have been originated for two reasons to explain the large difference of average velocities between red cells and plasma (the velocity of the red cells was calculated to be more than 2 5 times that of plasma) and to explain among other phenomena the variation of the extraction ratios of PAH and Diodrast

The calculated average velocity of the red cells in this investigation is 1 8 times that of plasma in cats and 1 5 in rabbits This difference in velocities between the red cells and plasma may be explained entirely by the axial flow (FÅHRAEUS 1929) and does not necessitate the existence of a short and long vascular pathway Another explanation of the velocity difference is discussed below

Without knowing any details about the diversion of the blood flowing from the glomeruli it may be concluded however that the outer portion of the peritubular vascular system receives blood from the outer glomeruli and the inner portion from the inner glomeruli According to the topography of the vessels it is probable that the blood through the outer portions has to travel a shorter distance than that through the inner portions This should accentuate the difference in the average velocities of cells and plasma if a separation occurs in the interlobular arteries Thus there seem to be several possible reasons for the velocity differences between red cells and plasma

KINTER and PAPPENHEIMER (1956 b) found that the extraction ratio of PAH and Diodrast diminished with decreasing hematocrit and this was confirmed by THOMPSON *et al* (1957)

The former authors suggested a by pass of plasma through the postglomerular shunts during low arterial hematocrit thus partly depriving the tubular cells of their supply of PAH The latter authors did not believe in an intrarenal cell separation but could offer no explanation of the differences of extraction ratios

If we accept the existence of a separation in the interlobular arteries the following explanation may suffice without requiring a postglomerular separation

The excretion capacity of the outer nephrons is smaller than that of the inner nephrons When the arterial hematocrit is low the supply of PAH or Diodrast to the outer part of the cortex is large and exceeds the transfer capacity of the outer tubules and thus the extraction ratio of the total kidney will be lower at low than at high red cell concentrations

5 *Distribution pattern during different functional conditions* Very little is known about the amount of blood flowing through different regions of the kidney KRAMER and collaborators (1960) have worked out a photometric method for measuring red cell and plasma flow in the outer cortex and the inner medulla They found that not more than [illegible] per cent of the total renal blood

flow passes through the inner medulla of the dog kidney. The lack of an autoregulation of the blood flow in the inner medulla is a very interesting result. It should be mentioned that one consequence of the cell separation theory will be a lack of autoregulation in the medulla.

THURAU *et al.* (1960) found an increased medullary blood flow during both osmotic and water diuresis and THURAU (1959) explained this as being due to a decreased viscosity of the blood in the papilla during diuresis. His explanation is based on the increasing albumin concentration towards the tip of the papilla found by LASSEN *et al.* (1958) and on the findings of GOTTSCHALK and MYLLE (1959) of equal osmotic pressures in medullary blood and urine. In the concentrating kidney the osmotic pressure of the blood in the papilla should increase and thus also the viscosity. Contrary to the findings of LASSEN *et al.* no plasma concentration gradient seems to have been found in this study either in diuretic or antidiuretic states.

It is of course very doubtful whether it is possible to correlate the blood flow with the blood volume in an organ. But nevertheless it should be discussed, whether the blood volumes found here in different functional conditions are compatible with the only two previous investigations on the determination of the size of the blood flow in the medulla at varying diuresis (THURAU *et al.* 1960 and LILIENFIELD, MAGANZINI and BAUER 1960). The marked increase in blood volume during water diuresis may indicate an enhanced blood flow in the medulla. Such an assumption becomes more plausible with the knowledge that THURAU *et al.* found a greater medullary blood flow during water diuresis. On the other hand I found no differences in the blood volumes of the kidneys during osmotic diuresis and antidiuresis while THURAU *et al.* found an enhanced blood flow during osmotic diuresis. LILIENFIELD *et al.* (1960) however arrived at the opposite result.

The problem concerning the blood flow in the medulla — and other parts of the kidney — is not yet solved and will without doubt be subjected to numerous investigations, in particular regarding the counter current mechanism and the autoregulation of the blood flow.

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Hematocrit and Hemoglobin Concentration in Venous Blood Drained from the Outer Cortex of Cat Kidney

By

HANS R ULFENDAHL

Received [illegible] January 1962

Abstract

ULFENDAHL, H R *Hematocrit and hemoglobin concentration in venous blood drained from the outer cortex of cat kidney* Acta physiol scand 1962 56 61—69 — The cell separation theory of PAPPENHEIMER and KINTER for the intrarenal blood flow was studied in cats by measuring red cell concentration in subcapsular veins on the renal surface and in arterial blood The venous blood taken from the subcapsular vein was assumed to be drained from the outer parts of the cortex. Hematocrit values and hemoglobin concentrations were significantly higher at blood pressures above 75 mm Hg in blood from the subcapsular veins than in arterial samples Below 75 mm Hg no difference was found Control experiments showed that the most probable explanation of the high red cell concentration in blood draining the outer part of the cortex was the occurrence of an intrarenal separation of red cells and plasma but the extent of separation was not clear

PAPPENHEIMER and KINTER s cell separation theory for the blood circulation in the kidney based upon a pure hemodynamic concept postulates a separation of red cells and plasma in the interlobular arteries (PAPPENHEIMER and KINTER 1955 and 1956 KINTER and PAPPENHEIMER 1956 a and b) Thus the inner glomeruli receive a cell poor blood and the outer glomeruli a cell rich one As the medulla is nutrified from the inner glomeruli it will get the cell poor blood. Also postulated was a postglomerular diversion of red cells and plasma into separate channels, a short-circuit for the red cells and a long-circuit for the plasma

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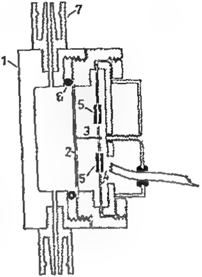

Fig 2 The design of the pressure transducer 1 Pressure chamber made in Perspex 2 phosphor bronze membrane 3 metal pin connecting the two bronze membranes 4 second phosphor bronze membrane 5 four strain gauges cemented to the membrane (4) 6 rubber packing ring 7 Luer lock male connection All lined parts are made of chromium plated brass

An exposed subcapsular vein was punctured with a knife and blood could freely flow out on to the surface of the kidney In some cases the vein was cannulated with a small glass capillary and blood was taken from the capillary for analysis The back flow from the renal vein was prevented by the obstruction of the actual subcapsular vein by the method of heat coagulation Fig 1 shows the anatomical conditions the straight arrows indicate the obstructed parts of a subcapsular vein

Before the vein was punctured it was controlled that blood from the surrounding tissues was not streaming to the region of blood sampling or that no tissue fluid was squeezed out from the renal tissue

To obtain arterial blood samples one of the carotid arteries was cannulated with a polyethylene tube

To be able to vary the blood pressure in the renal artery an adjustable clamp was placed on the abdominal aorta just under the diaphragm In other experiments the blood pressure was reduced by bleeding the animals High pressures were induced by injection of blood from a donor cat or by injection of dextran

The mean arterial blood pressure was registered in a femoral or mesenteric artery For the recording a pressure transducer of a strain gauge type[1] a DC-amplifier (Radiometer PHM 22) and an ink recorder (Varian G 10) were used

In some of the experiments no arrangement was made for influencing diuresis while in other experiments an osmotic diuresis was induced by intravenous injection of 0 5 g urea and 0 5 g glycine In some experiments adrenaline was given i v in order to raise the arterial blood pressure

[1] The pressure transducer was built in the Institute and its construction is seen in Fig 2 Four strain gauges (Philips PR 9711 120 ohm) were cemented to the second phosphor bronze membrane two on each side and were connected in a Wheatstone bridge which was supplied with 2 volts DC This very cheep transducer is however not suitable for dynamic measurements due to its high volume displacement (75 mm /100 mm Hg) The linearity varies from one transducer to another but is generally satisfactory up to 300 mm Hg

Table I

Experimental conditions	The percentage difference between hematocrit, Hct, or hemoglobin concentration, Hb of blood from subcapsular vein and arteries			
	BP < 75 mm Hg		BP > 75 mm Hg	
	Mean	Significance of the difference	Mean	Significance of the difference
Basal urine secretion, HCt	−1 3	P > 0 1	7.2	P < 0 001
Basal urine secretion Hb	0 3	P > 0 1	9 8	P < 0 001
Osmotic diuresis, Hct	0 1	P > 0 1	3 7	0 001 < P < 0 01
After injection of adrenaline Hct	—	—	7 1	P < 0 001

Table II

	The percentage difference between hematocrit and hemoglobin concentration of venous renal blood and arterial blood			
	BP < 75 mm Hg		BP > 75 mm Hg	
	Mean	Significance of the difference	Mean	Significance of the difference
Hemoglobin concentration	2.5	P > 0 1	−2 3	P > 0 1
Hematocrit	−3 4	0 01 > P > 0 001	−5.2	P < 0 001

> 0 01) At blood pressures below 75 mm Hg the differences decrease with decreasing blood pressure.

In the experiments with osmotic diuresis the results were similar to those in the experiments with basal urine secretion mentioned above (Table I)

Even after raising the blood pressure with adrenaline there was a hematocrit difference between blood from the two sources This in spite of the likely decrease in renal blood flow by constriction of renal vessels

In the experiments where blood from both the large renal vein and a carotid artery was analysed the Hb concentrations from the two sources were found to be the same. This was however not the case with the hematocrit that showed lower values *in the renal vein at both high and low arterial blood pressures* The results are shown in Table II

Discussion

According to v. MÖLLENDORFF and SCHRÖDER (1930) the cortical venous blood in cat kidneys flows away through two separate venous systems. The inner part of the cortex is drained through the profound cortical veins into the arcuate veins and the outer part through the superficial cortical veins into the subcapsular veins.

Thus the venous blood obtained by the puncture of these subcapsular veins, is assumed to be representative for the outer cortex.

The objection will perhaps be made that the puncture of the vein changed the hemodynamic conditions due to the following reasons. If the puncture caused a decrease in venous pressure in the actual vein then there should consequently be an increased blood flow in the adjacent part of the outer cortex and the blood flow should partly be diverted from the inner cortex to the outer one through the anastomosis between the profound and the superficial venous systems. Thus the blood taken from the subcapsular veins should not be considered as being derived from the outer cortex only, but also partly from the inner cortex. Such a diversion of the blood flow caused by the experimental technique should imply that the differences in hematocrit and Hb concentration between the superficial blood and the arterial blood in intact kidneys are greater than those found in the present experiments.

Factors causing the difference in Hb concentration between arterial blood and blood from the outer cortex. A hemoconcentration takes place when blood is passing the glomeruli. The volume of the glomerular filtrate is about 20 per cent of the plasma volume flowing into the glomeruli which means little more than a 10 per cent reduction of the blood volume. But about 85 per cent of the filtered volume is assumed to be reabsorbed in the proximal tubules (WALKER *et al.* 1941) and thus the net reduction of blood volume is decreased to 1 or 2 per cent. The actual volume reduction is perhaps still further reduced by a reabsorbtion of water in the distal tubules.

The hemoconcentration by the lymph flow is also very small and can be neglected (LEBRIE and MAYERSON 1960).

The most plausible explanation of the larger Hb concentration of the venous blood from the outer cortex seems to be the occurrence of a separation of red cells and plasma in the interlobular arteries.

Factors causing the difference in hematocrit between arterial blood and blood from the outer cortex. The hematocrit differences may depend upon the following factors:

1. Decreased water content of the blood after filtration in the glomeruli.
2. Change of ion concentrations.
3. Increased pCO_2 and decreased pO_2.
4. Cell separation in the interlobular arteries.

ad 1. The influence of this decrease must be small for the same reason as discussed above for the Hb concentration.

ad 2 The change of ion concentration and osmolarity of the blood during its passage through the cortex is apparently, small according to several investigations on the ion concentrations in the cortex (WIRZ 1956, GOTTSCHALK and MYLLE 1959 and BRAY 1960)

ad 3 The experimental data given by JACKSON *et al* (1954) showed that the hematocrit of the blood was not influenced to any greater extent, when the pO and pCO_2 were changed within the physiological limits, however at high pCO_2 and low pO_2 they found a swelling of the red cells Due to the very large blood flow through the kidney at blood pressures over 75 mm Hg, there would seem to be only small differences in pO_2 and pCO_2 between the venous blood and the arterial blood in the cortex and, thus, the hematocrit differences found in this investigation cannot be explained by a swelling of the red cells Moreover the swelling should increase with decreased blood pressure which did not happen

SWANN *et al* (1956) studied the hematocrit of the renal venous blood and found it to be the same as that of arterial blood Further the control experiments of this investigation did not show any larger differences in hematocrit or Hb concentration (Table II)

ad 4 The occurrence of a separation of red cells and plasma is then the only remaining possible explanation for the higher hematocrit found in the superficial venous blood at a high arterial blood pressure

The extent of the cell separation PAPPENHEIMER and KINTER (1956 a) postulated a large difference in hematocrit between the blood supplying the most super and the most profound glomeruli With an arterial blood hematocrit of 145 their postulated values for the blood in the most superficial and the most profound glomeruli were 0 80 and 0 10 respectively From these values it is possible to calculate the average hematocrit of blood flowing from the outer and the inner half of the cortex if the hematocrit at the border between the two halves is assumed to be 0 45 they are found to be 0 63 and 0 28, respectively This gives a difference of 0 18 between the blood from the outer cortex and the arterial blood. The quotient of this difference and the arterial hematocrit is 0 39 The same quotient in this investigation is about 0 10 at high blood pressures but this quotient may be too low due to the diversion of blood discussed above It must on the other hand be pointed out that the values of PAPPENHEIMER and KINTER are hypothetical and that they may be too high.

Even if the results of this investigation partly support the cell separation theory, little can be said about the influence of the cell separation on the renal function It may be that PAPPENHEIMER and KINTER have overestimated the importance of their theory but on the other hand it is impossible to agree with other authors who totally deny cell separation and its consequences Thus the discussion of the quantitative importance of cell separation for renal physiology is still highly speculative.

My thanks are due to Cand Med SVETOZAR KUSIC and Mr KARL-ERIK JOHANSSON for invaluable assistance

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Autonomic Nervous Control of Uveal Blood Flow

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Abstract

BILL A *Autonomic nervous control of uveal blood flow* Acta physiol scand 1962 *56* 70—81 — A study was made of the influence of the autonomic nervous system and its transmittor substances on the vascular resistance *UVR* influencing the blood flow through the uvea in rabbits and cats The results were analyzed and interpreted on the basis of an α β and γ receptor concept Electrical stimulation of the cervical sympathetics produced considerable increments in *UVR* in both species indicating α receptors Isopropylnoradrenaline given intraarterially did not change *UVR* in any of the species Neither did i a administration of adrenaline after pretreatment with dibenzyline There was then no indication for β receptors Acetylcholine given i a reduced *UVR* in both the species indicating γ receptors These receptors could be activated neither by stimulation of the ciliary ganglion (experiments only in cats) nor by stimulation of the cervical sympathetics The γ receptors therefore seem not to be innervated by any of these routes Results indicate that the flow reactions in the choroid were about the same as those within the anterior uvea

The autonomic nervous system and its transmittor substances influence the major vascular beds in the body in a way that is not clearly understood It has been pointed out however that many apparently contradictory findings might be explained on the basis of an α β- and γ receptor hypothesis (AHLQUIST 1948 1958 GREEN and KEPCHAR 1959) It should be pointed out, that the receptor concept is a working tool and does not refer to any anatomical structures

Alpha receptors, according to the receptor hypothesis produce vasoconstriction They respond to adrenergic drugs and are considered to be innervated by the sympathetics as they may be activated in some way by sympathetic

stimulation *Beta* receptors, which are not innervated produce vasodilatation for adrenergic drugs Finally *gamma* receptors produce vasodilatation and respond to cholinergic substances Some γ receptors are innervated that is they may be activated by cholinergic sympathetic or parasympathetic fibres while others seem to lack innervation

Great differences in distribution of t e receptors have been reported (For review see GREEN and KEPCHAR 1959)

The purpose of the present work was to study the uveal vascular bed in rabbits and cats with regard to the α β and γ receptor concept It was of interest to determine which of the virtual receptors are present in the different major parts of the uvea how they can be stimulated and finally to make a rough determination of their importance for the vascular resistance *UVR* influencing the blood flow through the uvea

UVR was defined as *(MAP IOP)/I* where *MAP* = the mean arterial blood pressure (in a femoral artery), *IOP* the intraocular pressure and *I* the uveal blood flow The pressure in the arteries entering the uvea is definitely lower than that in a femoral artery (see DUKE ELDER 1926 SEIDEL 1937), while the pressure in the veins leaving the uvea under most conditions approximately equals the *IOP* (BILL 1962 a b) Therefore the vascular tree the receptors of which were investigated includes the uveal vessels and some of the extra ocular part of the arteries supplying the uvea but not the extraocular veins

Methods

The animals employed were cats weighing 1 8—3 4 kg and albino rabbits weighing 1 8—2 9 kg

Cats were anesthetized with chloroform inhalation and anesthesia was maintained with chloralose urethane given intravenously (proportions 1 10) Rabbits were anesthetized with a barbiturate (Veterinary NembutalO Abbott initial dose 0 75 ml/kg body weight) given i. v and anesthesia was maintained with small additional doses given when required In the search for possible β and γ receptors some experiments were performed with anesthesia induced and maintained with urethane (initial dose 1 0—1 3 g/kg body weight) In most experiments in rabbits the eye was also anesthetized locally during the preparation procedure (4 per cent cocaine in isotonic saline)

Tracheotomy tubes were inserted in all animals to ensure a patent airway The animals were placed horizontally on one side Heparin (1 500 U/kg body weight) was given i v to prevent coagulation of blood and aqueous humour

The anterior chamber was cannulated and in most experiments, the intraocular pressure was stabilized at 20—25 mm Hg using a levelling manometer The actual intraocular pressure could be determined with a pressure transducer (Elema EMT 490 A, volume displacement 0 07 mm³/100 mm Hg) The normal intraocular pressure in rabbits and cats maintained under general anesthesia is about 20 mm Hg (BECKER and CONSTANT 1956)

The mean arterial blood pressure was measured in a cannulated femoral artery by means of a pressure transducer of the same type as described above

The blood flow through the uvea was determined with both indirect and direct methods described in detail elsewhere (references below)

For *direct* determinations of the blood flow from the uvea in *cats* almost the whole uvea was made to drain into a polyethylene tube introduced into an intrascleral venous plexus This gives a marked redistribution of the blood flow from the eye but changes the state of the vessels within the eye very little (BILL 1962 c)

For *direct* determinations of the blood flow through the uvea in *rabbits* one of the four vortex veins was opened and in some experiments cannulated The total uveal blood flow was determined as four times the flow from the opened vein Under most conditions this procedure is adequate (BILL 1962 a b)

For *indirect* determinations of changes in choroidal blood flow in *rabbits* and *cats* a calorimetric procedure was used The method was a modification of the heated thermocouple principle of GIBBS (1933) and its application for studies in eyes was reported earlier (BILL 1962 d e)

When the blood flow was determined with direct methods the blood collected was returned to the animal via a femoral vein Losses of blood from the system were compensated for by intravenous infusions of a dextran solution (Vetridex©, Pharmacia) Graphic recorders (Varian G 10) were used for all registrations

In the study of the α receptors electrical stimulation was performed either with a stimulator constructed by CATTON MOLYNEUX and SCHOFIELD (1957) or an AEL Laboratory stimulator (American Electronic Laboratories Model 104 A) With the former stimulator, frequency and voltage could be varied the pulse shape was part of condensor discharges With the latter stimulator rectangular wave pulses were obtained. Frequency voltage and duration could be varied In the search for γ receptors only the AEL stimulator was used

Platinum electrodes (inter electrode distance 2 mm) were used to stimulate the sympathetics The electrodes were attached to the superior cervical sympathetic ganglion on the side of the eye under study or to the sympathetic chain just below the ganglion The chain was cut some millimeters below the lower electrode (It should be mentioned that some minor part of the sympathetic fibres supplying the eye do not pass through the structures stimulated See HOLLAND VON SALLMAN and COLLINS 1957) For stimulation of the ciliary ganglion silver electrodes (inter electrode distance 1 mm) were employed The ganglion was stimulated only in cats It was exposed and isolated from its central connections in a manner essentially the same as that described by ARMALY (1959) In all stimulation experiments the stimulation was continued until flow attained a rate which was approximately steady for 1 min

In some experiments for i a injections an omega shaped polyethylene tube was interconnected in the common carotid on the side of the eye under study Injections were performed through a second polyethylene tube introduced through the wall of the former tube with its tip pointing against the blood flow direction In other experiments the superior thyroidal artery was cannulated and injections made through this vessel into the common carotid To determine the adequacy of these techniques Evan's blue was injected with the same speed as the agent under study and it was observed if it reached the eye

Investigation of α receptors Electrical stimulation of the cervical sympathetics was the procedure used to reveal the existence of α receptors The intensity and duration when adjustable were regulated to give maximum responses at the different frequencies employed As a rule 3—7 volts and 1—5 milliseconds proved suitable

Investigation of β receptors Two procedures were used in the search for these receptors One was to give l isopropylnoradrenaline i a without previous blockade of α receptors This drug affects essentially β receptors (see GREEN and KEPCHAR 1959) The other was to give adrenaline i a after blockade of α receptors with 5—50 mg/kg body weight of dibenzyline (phenoxybenzamine)

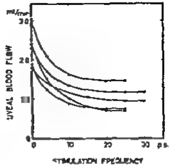

Fig 1 The effect of stimulation of the cervical sympathetics on uveal blood flow in rabbits The uveal blood flow was determined as four times the flow from an opened vortex vein.

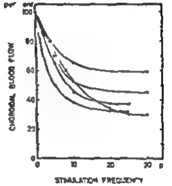

Fig 2 The effect of stimulation of the cervical sympathetics on the blood flow through the choroid in rabbits The choroidal blood flow was determined with a calorimetric procedure The flow values are given as per cent of the flow present before the stimulation was started.

Investigation of γ receptors Acetylcholine was given i. a to demonstrate the presence of γ receptors Vasodilator cholinergic sympathetic fibres were searched for after dibenzyline blockade of the α receptors For technical reasons vasodilatator fibres in the orbital parasympathetics were looked for only in cats The ciliary ganglion contains both sympathetic and parasympathetic fibres The constrictor effects of the former were blocked by reserpine pretreatment. ROSELL and ROSEN (1961) have shown that after such treatment stimulation of nerves containing both adrenergic and cholinergic fibres produces essentially cholinergic effects The reserpine was given subcutaneously for 2—3 days before the actual experiments in doses of 1—4 mg/kg body weight.

In the search for innervated γ receptors stimulation intensity was varied from 1 to 20 volts duration from 0 1 to 20 msec and frequency from 1 to 100 pulses per second

Results

α receptors

Sympathetic stimulation in rabbits In five eyes with artificially stabilized intraocular pressure *total uveal blood flow* was determined at stepwise increased stimulation frequencies

In each experiment the flow decreased when the sympathetics were stimulated the effect increasing with increasing stimulation frequency until a frequency of about 25 per second was reached. A further increase in frequency did not further reduce the flow Fig 1 presents the results obtained.

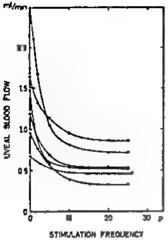

Fig 3 The effect of stimulation of the cervical sympa thetics on uveal blood flow in cats

The mean increase in *UVR* caused by a stimulation frequency of 25 pulses per second was 136 $\pm$ 29 per cent

In 12 eyes the relationship between *local choroidal blood flow* and the stimula tion frequency was determined, and 5 experiments were acceptable from a technical point of view Fig 2 presents the results The mean increase in vascular resistance opposing flow through the choroid, as determined by analogy to *UVR* was 161 $\pm$ 30 per cent There was thus no essential difference in the eaction to maximum sympathetic stimulation between the vessels of the an erior uvea and those of the choroid

Sympathetic stimulation in cats In 6 eyes the *total uveal blood flow* was measured .t stepwise increased stimulation frequencies at a stabilized intraocular pres ure The uveal blood flow decreased with increasing stimulation frequency vithin the same frequency range as in rabbits, as demonstrated in Fig 3 The nean increase in *UVR* caused by a stimulation frequency of 25 pulses per econd was 138 $\pm$ 16 per cent In two experiments qualitative changes in ocal choroidal blood flow were also determined These experiments showed hat the *choroidal blood flow* changed in the same direction as total uveal blood low during the stimulations

β receptors

I a injections of isopropylnoradrenaline at a stabilized intraocular pressure did not change the blood flow through the uvea as long as the arterial blood pressure was constant Doses large enough to cause a reduction in arterial blood pressure gave a decrease in uveal blood flow secondary to the pressure fall. The drug was administered in doses increasing from 0 1 μg given over 3 min to 100 μg given over 10 sec

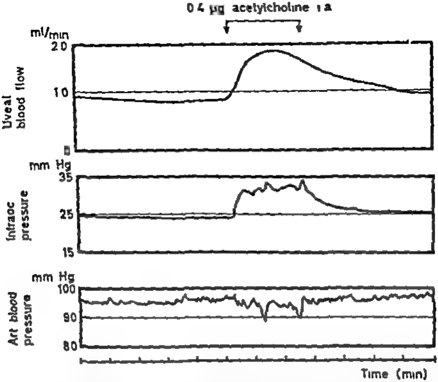

Fig 4 A. Responses in uveal blood flow intraocular pressure and arterial blood pressure to acetylcholine injected into the common carotid supplying the eye under study The uveal blood flow was determined as four times the flow from an opened vortex vein Experimental animal rabbit.

Adrenaline given i a in doses from 0 1 μg over 3 min to 100 μg over 10 sec did not essentially change *UVR* after administration of dibenzyline although adrenaline reversal in blood pressure reactions was produced in all experiments In these experiments acetylcholine injected i a produced vasodilatation which made it certain that the dibenzyline had not deprived the uveal vessels of all tone In rabbits 7 eyes were investigated for β receptors in cats 8 eyes

γ receptors

Acetylcholine and uveal blood flow in rabbits Acetylcholine given i a in doses of 0 01 μg produced an increase in *total uveal blood flow* in all of 5 eyes investigated The largest increases were obtained with doses of about 0 5 μg given over 2—3 min There was then an increase in uveal blood flow of about 100 per cent in several experiments this in spite of a simultaneous fall in arterial blood pressure *UVR* was thus reduced to less than 50 per cent of the original value The change occurred irrespective of whether the intraocular pressure was

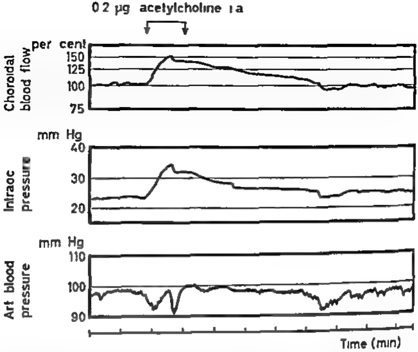

Fig 4 B Responses in choroidal blood flow intraocular pressure and arterial blood pressure to acetylcholine injected into the common carotid supplying the eye under study The choroidal blood flow was determined with a calorimetric procedure The flow values are given as per cent of the flow present before the injection commenced Experimental animal rabbit

h or not Fig 4 A presents the result of an experiment in which the intraocular pressure was not stabilized

In 5 eyes the reaction in *local choroidal blood flow* was determined Also in these experiments acetylcholine produced increases in blood flow which were of the same order as in the above experiments Fig 4 B

Acetylcholine and uveal blood flow in cats In cats acetylcholine given i a and in the same doses as in rabbits produced large reductions in *UVR* Similarly to the condition in rabbits the changes were rather variable However, 0 5 µg given over 2—3 min as a rule reduced the resistance to less than 50 per cent of that initially present Reductions of up to 25 per cent of the initial values were produced in [illegible] of the eyes Fig 5 shows a typical result In all 5 eyes were investigated In 3 experiments the effect of acetylcholine on *local choroidal* blood flow was investigated and the drug was found to produce a considerable increase in flow

Innervated γ receptors in rabbits and cats In neither of the species did stimulation of the cervical sympathetics after blockade of α receptors produce a decrease in *UVR* In spite of very large doses of dibenzyline the usual response

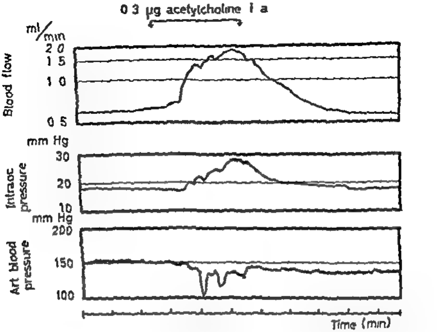

Fig 5 Response in uveal blood flow intraocular pressure and arterial blood pressure to acetylcholine injected into the common carotid artery supplying the eye under study Experimental animal cat

was a rather slight increase Experiments were performed in 5 rabbits and 5 cats

Stimulation of the ciliary ganglion in cats produced contraction of the pupil even to intense miosis without notably changing *UVR* These experiments were performed in 7 eyes

There was thus no evidence for either sympathetic or parasympathetic innervation of the γ receptors found in the uvea

Discussion

In rabbits the mean arterial blood pressure at the origin of the ciliary arteries is about 15 mm Hg lower than that in a femoral artery (unpublished observations) In cats the pressure in the ophthalmic artery is to much the same degree lower than that in the aorta (Duke Elder 1926) The difference in pressure is no doubt influenced by flow through tissues supplied by the same arteries as the uvea Changes in vascular resistance in these tissues therefore will tend to alter the pressure in the ciliary arteries and thereby the uveal blood flow It seems probable however that normally the ciliary arterial blood pressure is little influenced by this mechanism as intense vasoconstriction within

ELDER (1931) to be restricted to the capillaries The present findings indicate that, if present, β receptors within the uvea have very little influence on the blood flow under the conditions of study It seems possible that some of the effects observed by the earlier investigators were, in fact, due to venoconstriction.

γ receptors The earlier literature concerning the effect of the parasympathetics on the uveal vessels was very indefinite There was not even agreement on the effect of acetylcholine (ACh) on the vascular resistance within the uvea

COLLE et al (1931) working with dogs reported an increase in intraocular pressure after administration of ACh to the uveal vessels and this was looked upon as evidence for a vasodilatatory effect of the drug WUDKA and LEOPOLD (1956), however in rabbits found no change in the diameters of the larger choroidal vessels resulting from administration of cholinergic drugs, which was regarded as evidence against a vasodilatatory effect of such drugs on the uveal vessels These authors may also be consulted for a review of the earlier literature

The present findings demonstrate conclusively that ACh reduces the vascular resistance controlling uveal blood flow The negative findings of WUDKA and LEOPOLD, quoted above, make it seem likely that ACh, as in the case with sympathetic stimulation affects essentially the vessels in the deeper layers of the choroid and those within the anterior uvea

The fact that stimulation of cholinergic fibres to the uvea did not produce vasodilatation suggests that normally ACh liberated within the eye does not reach the uveal vessels sensitive to it

It was somewhat surprising that the large changes in iris position produced by stimulation of the ciliary ganglion did not cause a change in the blood flow [illegible]gh the uvea There is little reason, however to suspect that iris contrac[illegible] reduced the blood flow through this part of the uvea masking an increase in flow through other parts

Fig 6 presents schematically the receptors found in the present study and the autonomic nerves supplying the uvea

The present work has been supported by grants from the Medical Faculty in Uppsala and by a grant (B 3060) from the United States Public Health Service Part of the equipment used was placed at the author s disposal from the Department of Ophthalmology University of Uppsala.

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Acta physiol. scand. 1962. 56. 82—89

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Continuous Recording of Arteriovenous Differences in Concentration of Radioactively Labelled Substances

By

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Received 3 February 1962

Abstract

BERLIN, M. *Continuous recording of arteriovenous differences in concentration of radioactively labelled substances.* Acta physiol. scand. 1962. 56. 82—89. — A unit has been designed for continuous measurement of differences in concentration of radioactively labelled substances between two fluid systems. A transistorized difference ratemeter was used. Tests and calculations were performed in order to obtain an impression of the measuring characteristics of the apparatus. The unit appears to be suitable for continuous measurement of arteriovenous concentration differences in biologic tracer experiments.

Continuous measurement of the radioactivity of blood by means of scintillation detectors or Geiger Muller tubes is a well tried procedure. Practical difficulties are encountered, however, in measuring arteriovenous (a-v) differences in radioactivity. If the arterial and venous intensities are measured with two separate detectors and the difference is calculated from single readings on the ratemeter recordings, the accuracy will be low. Moreover, adjustment and calibration of the two channels are tedious and time-consuming. With the apparatus described in this paper an attempt has been made to improve the accuracy of, and to simplify the procedure for such measurements by incorporating a specially designed difference ratemeter. The method is applicable to radiation pulses measurable with solid scintillators. In order to test the accuracy and speed of response of the apparatus, and thus to obtain an impression of its characteristics, various tests and calculations were performed.

Electronic apparatus for measuring radioactivity

Fig. 1 gives a diagramatic presentation of this apparatus. The arterial and the venous blood is pumped through polythene spirals placed *in* or *above* the crystals in scintillation detectors. The pulses from the detectors are amplified and led to discriminators. The pulse frequency from the detector for arterial

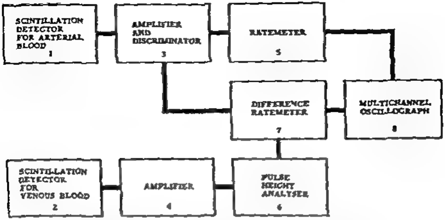

Fig 1 Block diagram of the apparatus.
1 I D L — Scintillation Counter head Type 653 with a plain phosphor 44 mm x 25 mm.
2 I D L — Scintillation Counter head Type 653 with a well type phosphor 44 mm x 51 mm with the hole 19 mm x 27 7 mm.
3 I D L — Wide Band Amplifier Type 657
4 Nuclear Enterprises ltd — Fairstein Non overloading Linear Pulse Amplifier Type NE 5202
5 Philips-type PW 4042
6 Nuclear Enterprises ltd. Singlechannel differential Pulse Height Selector NE 5102
7 See text.
8 Honeywell 906 Visicorder

blood is measured by a ratemeter The difference between the pulse frequencies from the detectors in the respective channels is computed by a specially designed difference ratemeter The ratemeter amplitudes are registered by a multichannel oscillograph with optical recording on direct developing photographic paper In principle well crystals and pulse height analyzers should be used for both channels but for the present purpose satisfactory results were obtainable with different types of crystals amplifiers and discriminator systems

The ratemeters and the recorder are calibrated by means of 50 cps pulse generators built into the meters The sensitivity of the scintillation detectors is matched and calibrated by filling the spirals with a standard radioactive solution — intensity up to 10 times the level to be measured — and adjusting the position of the spiral in the well crystal until the detectors are equally sensitive. The background levels for the difference ratemeter and the arterial channel meter are determined by recordings before and after each measurement.

The difference pulse ratemeter

The difference in pulse frequency between two sources may be measured with digital technique when high accuracy will be obtained This method is comparatively expensive however If the difference is formed from analogue representations the respective pulse rates will be measured with an accuracy of a few per cent and the accuracy of the difference pulse rate will be low espe-

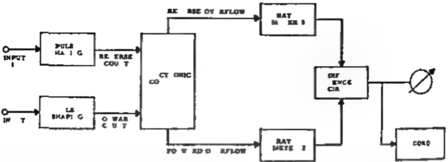

Fig 2 Block diagram of the difference ratemeter

cially if the input frequencies are of similar magnitude The described difference ratemeter utilizes combined digital analogue technique and gives reasonable accuracy at reasonable cost Solid state elements (transistors etc) are used throughout the apparatus

A simplified block diagram of the ratemeter is shown in Fig 2

Pulses from the two detectors are fed to inputs 1 and 2 of the apparatus The counter, which consists of two bistable multivibrators and a diod matrix serves as a memory with a capacity of three pulses Pulses on input 2 fill the memory and pulses on input 1 empty the memory When the memory is empty, pulses on input 1 pass to ratemeter 1 Similarly when the memory is full, pulses on input 2 pass to ratemeter 2

It should be noted that when more than three pulses are received on the lower rate input between two consecutive pulses on the higher rate input, a will be sent to the ratemeter which normally is nonoperative This means cancellation of a few pulses will take place after the ratemeters, where the iracy is inferior Such a situation occurs very rarely however and should t affect the overall accuracy to a notable degree

If pulses reach the counter from the two inputs with a time difference less an the recovery time for the counter unit one pulse is lost The possibility of ch a coincidence is slight however Moreover the pulse loss will affect both put channels to approximately the same degree The influence on overall accuracy should consequently be negligible

Accuracy and reproducibility of radioactivity measurements

The precision with which the radioactivity of the arterial blood and the a v difference in radioactivity can be determined is dependant upon a number of factors These are the stability and recording characteristics of the electronic apparatus, the accuracy with which the tracings can be read the uncertainty associated with the randomness of the radioactive decay and the errors involved in calibrating and adjusting the apparatus

The total errors, including all the mentioned factors for both channels were studied in 9 experiments in which the spirals in the detectors were filled with

Table I Deviation from expected value in the arterial channel ( )

Deviation	Test no									Mean deviation
	1	2	3	4	5	6	7	8	9	
Period 1	−5.5	+1 3	−1 5	−6.5	−6 0	−6.9	−4 9	−1 8	−3 0	−3.9
Period 2	−5 9	+2.8	−1.9	−6.9	−4 4	−5 6	−7 8	−1 4	−5 0	

a solution of I^{131} The solution gave about 1,000 cpm in the ratemeter of the arterial channel and a three hour run was made In order to study the tendency to drift the recorded amplitude was evaluated for one period at the beginning and one at the end of each run The intensity level (1 000 cpm) of the I^{131}-solution was chosen because it was thought to lie in the lower part of the measuring range of the apparatus The radioactive intensity of the test solution was determined in each experiment in a well crystal Since the detectors were calibrated before the experiments the expected amplitude for the galvanometer of the arterial channel could be calculated The difference between expected and observed amplitude thus represents the sum of the errors for the measurement in the well crystal and the measurement in the tested channel As the size of the first error was known the difference constituted an index of the accuracy of that part of the apparatus Since the expected amplitude for the difference channel was zero the recorded deviation from zero was the error in difference measurement

The difference channel was also tested by filling only the spiral of the arterial detector with the test solution The deflection of the galvanometer was then recorded for 24 hours The deviation from the expected galvanometer amplitude was read for 10 parts of the tracings each representing a period of 20 min at intervals of 1 1/2 hours

The tracings were evaluated by measuring the distance from the baseline for 50 points 2 mm apart These 50 points represented a 25 minute recording corresponding to 10 cm on the record The standard deviation and the mean of the 50 readings were calculated The values for standard deviation were compared with the calculated deviation due to randomness of the radioactive decay The latter was calculated according to

$$\sigma = \sqrt{\frac{V}{2\,RC}}$$

where σ is the standard deviation, N the number of pulses per unit time and RC the time constant Similarly the standard deviation due to decay randomness for any point on the difference ratemeter curve is

Table II Deviation from zero in percent of the input signals in the ratemeters

Deviation	Test no.									Mean deviation
	1	2	3	4	5	6	7	8	9	
Period 1	−1 4	−1 0	−2 0	−1 7	−9 6	−0 7	+0 1	−2 6	−2.0	−1 5
Period 2	−1.5	+5 3	−2.0	+1 2	−4 4	+3 4	−3 9	+1 2	−4.5	

where N_1 and N_2 are the respective numbers of pulses to the inputs per unit of time The standard error of the mean of the spot values was calculated according to SCHIFF and EVANS (1936)

This method of reading the tracings was compared in a special experiment with another method (the level-estimating method) which involved the following procedure With a ruler a line was drawn to represent as exactly as practicably the mean level of a curve representing a 10 min record The distance between this line and the baseline gave the required mean

In all tests the detectors were calibrated and matched at equal sensitivity with a standard solution of known strength giving about 10 000 cpm in the ratemeters The background level of radioactivity was recorded before and after each test for 20 min For both channels it was between 400 and 500 cpm The time constant for the ratemeters was set to 30 sec and the charting speed of the recorder to 4 2 mm per minute One cm on the record thus represented 100 cpm from the ratemeter

of the tests

Table I shows the deviations from the expected readings in the arterial channel in nine experiments The mean deviation from the expected amplitude was 1 9 % and implies the presence of a systematic error as well as random errors The differences between the values for the two periods separated by a 2 1/2 hours interval are not indicative of any considerable drift tendency in the apparatus

The deviations from zero in the difference channel in the nine experiments, expressed in per cent of the activity in the detectors are shown in Table II Only in exp 5 was the deviation too large to be attributable to randomness of the radioactive decay The readings for the two periods provide no evidence of an appreciable drift tendency in the channel

The standard deviations for the tracings from the arterial and difference channels were closely correlated to the calculated scattering due to randomness of decay in radioactivity It was not possible to show by statistical methods any difference between these standard deviations and the values calculated for decay scattering

Fig 3 presents the two methods for evaluating the tracings the spot method and the level-estimating method, in an experiment with varying activity levels.

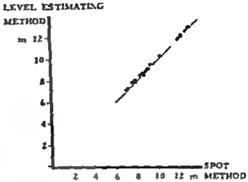

Fig 3 Comparison of the spot method and the level-estimating method in an experiment with varying activity levels

The values are closely correlated which indicates that little loss of accuracy is to be expected when mean values representing 10 minutes or more are determined with the level estimating method

Speed of response

The term "speed of response is here used to denote the capacity of the apparatus to reflect rapid changes in a v difference The speed of response varies inversely with the volume of the spirals and directly with the rate of flow through them — the more rapid the flow and the smaller the volume, the greater the speed of response The most important factor in this respect however is the time constant of the ratemeters which can be selected from the series 0 3 3 and 30 sec or 1 10 and 100 sec

If it is assumed that a ratemeter is in equilibrium when the amplitude is within 0 6745 σ from the true value the time to establish equilibrium can be obtained from the equation (Kip et al 1946)

$$t = RC \ (1/2 \ \log \ 2 \ NRC + 0\ 394)$$

The error is proportional to $\sqrt{N/RC}$ Therefore the lowest time constant that can be used is determined by the radioactivity of the blood and the required accuracy As the blood concentration of the substance under study will as a rule be dependent upon the purpose of the experiment the limiting factors for the speed of response are the sensitivity of the scintillation detectors and the specific activity of the labelling isotope

How the responsiveness depends on the damping in a ratemeter is illustrated in Fig 4 which shows the amplitude and duration of a square wave signal that produces a detectable change in the tracings under the conditions in the tests, i e at a level of activity giving 1 000 cpm and with a time constant of 30 sec A detectable change has been defined as one that exceeds 3 times the standard deviation at the activity level in question. The ratemeter can be considered as a simple RC circuit with the time constant RC. The charge I on the c

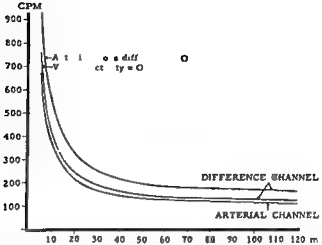

Fig 4 Relationship between amplitude and duration of a signal that can be detected in the arterial channel and in the difference channel when the difference is zero and in the difference channel when the difference is equal to the arterial activity (activity in venous channel = 0)

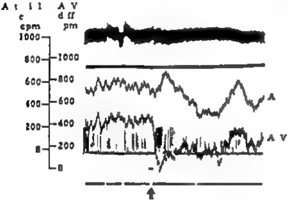

Fig 5 Record from an experiment in which the arteriovenous difference for I[131]-d odrast was measured across a rabbit kidney
A = arterial concentration of I[131]-diodrast
A V = arteriovenous difference
The arrow denotes an intravenous injection of inactive diodrast Remaining tracings represent from the top blood pressure temperature urine flow and time in minutes.

is then $V_0\,(1 - e^{-t/RC})$ where the potential difference across the circuit at time $t = 0$ is V_0 If $V = 3\,\sigma$ we have $V_0 = \dfrac{3\,\sigma}{1\ e^{-t/RC}}$ The diagram shows the relationship between the amplitude and duration of a signal that can be detected in the arterial channel and in the difference channel when the difference is zero, and in the difference channel when the difference is equal to the arterial activity (i e the activity in the venous channel is zero)

Examples of application of the method

Fig 5 shows the record from an experiment in which the a v difference for diodrast labelled with I[131] was measured across a rabbit kidney The upper ratemeter tracing shows the arterial concentration and the lower the a v difference for I[131] diodrast The decrease in difference is due to an intravenous injection of inactive diodrast which is indicated by the arrow

Comments

The tests showed that the observed fluctuations in the tracings from the arterial and the a v difference channels were in close agreement with the theoretic fluctuations due to the randomness of decay in radioactivity Nor was the accuracy appreciably lower than that usually specified for ratemeters The manufacturer states an accuracy within ± 3 per cent for the ratemeter used in the arterial channel The observed values support the view that the total accuracy, including calibration and matching procedures is well within ± 10 per cent The relevant figure is probably more than ± 3 per cent and less than ± 10 per cent.

In biologic material the variations as a rule are considerably greater than the errors with which we are concerned here The apparatus therefore would seem to be sufficiently accurate for purposes such as study of uptake and excretion of radioactively labelled substances under varying conditions in organs where withdrawal and return of blood can be effected by catheterization

In the tests of the apparatus I^{131} was used as radiation source Provided that the basic conditions in the tests are unaltered i e activity level in the detectors 1 000 cpm above background level and maximal background level 500 cpm the results of these tests can be applied to most other γ-emitting isotopes The tendency to discriminator and high tension drift might assume greater importance with isotopes having a γ ray energy spectrum differing from that of I^{131} In most cases the effect of the drift can be counteracted by suitable choice of voltages for the photomultipliers and discriminators

Probably little is to be gained in accuracy by raising the level of radioactivity The scatter due to the randomness of radioactive decay is not a major component of the total error By increasing the radioactivity the measuring range of the apparatus is altered but its accuracy is unaffected On the other hand the speed of response can be considerably increased since a rise in radioactivity permits a corresponding decrease in the time constant The possibility of accelerating response is limited however and for high efficiency in this respect it is necessary to use another method such as continuous drop collection which permits the measurement of radioactivity in each drop (SÖDERBERG 1958)

The author is indebted to Mr BENGT LINDBERG at The Royal Institute of Technology for the design of the difference ratemeter A grant from Reservationsanslaget of the Karolinska Institutet is gratefully acknowledged.

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Protective Effect of Bretylium on Noradrenaline Stores in Organs

By

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Abstract

RYD, G. *Protective effect of bretylium on noradrenaline stores in organs.* Acta physiol. scand. 1962. 56. 90—93. — The noradrenaline content of the liver, heart and kidney of the guinea pig was increased after an intraperitoneal dose of 50 mg bretylium, given 1/2 to 4 hours previously. No increase was found in the brain. In liver, spleen and heart the depleting action of reserpine on noradrenaline was partially prevented by bretylium, but no effect was noted in the kidney and brain. In organs analyzed 24 hours after injection of bretylium the amounts of catecholamines did not differ from the controls.

BOURA and GREEN (1959) found that bretylium specifically blocks the effect of adrenergic nerve stimulation, without interfering with the parasympathetic nerves. According to these authors the depressant action of bretylium is located peripheral of the sympathetic ganglia. The failure of adrenergic nerve stimulation to produce an action is not due to blocking of the effects of adrenaline or noradrenaline, since both amines show enhanced effects after bretylium as in the case of catecholamines after postganglionic neurosection. For these reasons it has been assumed that bretylium prevents the release of the neurotransmitter. After administration of bretylium for several weeks BOURA et al. (1959) found no depletion of the catecholamine content of tissues of cats, but GREEN (1960) has reported that some depletion occurs in spleen and heart of cats. FURCHGOTT and KIRPEKAR (1960) found no significant alteration of the catecholamine content of guinea pig atria after exposure to bretylium in acute experiments. The present experiments have been made in order to study whether the stores of catecholamines in various organs in vivo are influenced by bretylium in short term experiments.

Table I Noradrenaline μ_g/kg in guinea p g organs

Organ	Controls (n = 5)	½—4 hours after 50 mg/kg bretylium tosylate (n = 13)	Increase per cent	
Heart	1.3 ± 0.066	1.9 ± 0.11	46	P = < 0.001
Spleen	0.72 ± 0.094	0.98 ± 0.13	36	P = > 0.1
Kidney	0.34 ± 0.020	0.49 ± 0.051	44	P = < 0.02 > 0.01
Brain	0.29 ± 0.016	0.25 ± 0.029	—	—
Liver	0.17 ± 0.009	0.31 ± 0.035	[illegible]	P = < 0.01 > 0.001

Table II Noradrenaline in µg/g organ

Organ	Controls (n = 5)	24 hours after reserpine (n = 5)	After reserpine and bretylium (n = 7)
Heart	1.3 ± 0.066	< 0.05	0.17 ± 0.03
Spleen	0.72 ± 0.094	< 0.05	0.20 ± 0.05
Kidney	0.34 ± 0.020	0.02 ± 0.01	0.02 ± 0.01
Brain	0.29 ± 0.016	< 0.05	< 0.05
Liver	0.17 ± 0.009	< 0.01	0.03 ± 0.01

Methods

Bretylium tosylate was given intraperitoneally in aqueous solution in one dose of 50 mg/kg to guinea pigs weighing 300—500 g. The animals were sacrificed with a blow on the neck 1/2—4—24 hours after the injection. The organs were dissected out immediately, weighed and ground with a mixer after addition of 5 volumes 5 per cent trichloroacetic acid and extracted for 15 min. After filtering the residue was thoroughly washed with 2 volumes of 5 per cent trichloroacetic acid and the combined extracts adjusted to pH 8.3—8.4 with 1 N NaOH. The catecholamines were adsorbed on an alumina column and eluted with 5 ml 0.5 N followed by 5 ml 0.25 N acetic acid. The catecholamines were estimated fluorimetrically according to EULER and LISHAJKO (1961).

In a second series of animals bretylium was administered i. p. to guinea pigs in a dose of 30 mg/kg. One hour later 0.5 mg/kg reserpine (Serpasil® Ciba) was given, followed by two doses of bretylium tosylate (Darenthin, kindly given by Dr. A. L. A. BOURA, Beckenham) 20 mg/kg each, after 6 and 20 hours respectively. After 24 hours the animals were killed and the catecholamine content of the organs determined as described above.

Results

1. *Bretylium*

Table I indicates that the noradrenaline content is considerably increased in the liver, the heart and the kidney after the administration of bretylium. Also the spleen showed an increase of noradrenaline, on an average 35 per cent

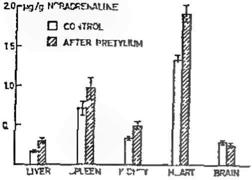

Fig 1 Noradrenaline content in various organs of guinea pig before (empty columns) and after bretylium 50 mg per kg intraperitoneally (striped columns) with standard error of the mean.

This increase, however, was not significant but it should be noted that the S E was high No effect of bretylium was noted on the brain Since no difference was observed between the group killed half an hour after the injection of bretylium and the one killed after four hours all values were treated together The administration of bretylium did not seem to have any consistent effect on the adrenaline content of the organs

In organs removed 24 hours after the injection of bretylium no difference in catecholamine values was found in comparison with the control figures

2 *Bretylium and reserpine*

The well known effect of noradrenaline depletion after reserpine is seen in Table II which also shows that the injection of repeated doses of bretylium to reserpinized guinea pigs counteracts to some extent the noradrenaline depletion caused by reserpine alone The above effect was noticed in the liver the spleen and the heart, whereas in the kidney and the brain no antagonistic effect of bretylium could be recorded Changes in the amount of adrenaline as a result of administration of bretylium to the reserpinized animal were observed only in the liver in which a small but statistically significant increase was observed.

Discussion

The finding that the amounts of noradrenaline in organs from guinea pigs can increase after the administration of bretylium suggests that the organ stores presumably in the adrenergic nerves are normally in a state of slight depletion. This might occur as a consequence of stimulatory influences chiefly of reflex origin perpetually releasing adrenergic transmitter substance as evidenced by the constant finding of noradrenaline in urine If the ensuing release from the adrenergic neurons is prevented for instance by bretylium, the storing process might be assumed to proceed up to the storage limits Since no differences were found between the noradrenaline values in organs analyzed 1/2 and 4 hours after the injection of bretylium it may be assumed that the storing process is

complete within a short time The results obtained by EULER and UDDÉN (1951) who found increased amounts of noradrenaline in several organs of the cat after administration of various catecholamine precursors may be interpreted in the same way

The lack of effect of bretylium on the catecholamine values in organs analyzed 24 hours after the injection indicates that the bretylium effect is of relatively short duration This is also in keeping with the results obtained in other kinds of experiments (BOURA et al 1959)

A possible explanation for the inability of bretylium to influence the catecholamines in the brain might be that bretylium does not pass the blood brain barrier Thus BOURA et al (1960) after injection of ^{14}C-labelled bretylium in cats found only a slight accumulation of this substance in the brain

The antagonistic action of bretylium on the depleting effect of reserpine on the catecholamine content in organs may be explained on the grounds that the gradual leakage of the amines from the stores after reserpine is slowed down by bretylium An antagonistic action of bretylium administered by the intraventricular route against reserpine has recently been reported by NORTON and COLVILLE (1961)

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Blocking of the Thyroid Response to Cold by Local Warming of the Preoptic Region

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Local cooling of the preoptic region was recently found to cause a conspicuous activation of the thyroid gland concomitant with the development of marked core hyperthermia (ANDERSSON *et al* 1962 a and b) On the basis of this observation it was postulated that the preoptic heat loss center (MAGOUN *et al* 1938) normally exerts a tonic inhibition of the release of thyrotrophic [hormo]ne from the pituitary Due to inactivation of the heat loss center during cold exposure and hypothermia this inhibition would be removed as a link in the metabolic response to cold Further evidence for the assumption would be provided if it could also be shown that local warming of the preoptic region blocks the thyroid response to cold, especially since this response has been shown to be mediated via the hypothalamo pituitary axis (C v EULER and HOLMGREN 1956) The study reported briefly in the following shows that such is really the case

By a special technique of high frequency warming between two bilaterally implanted silver plates, it was possible to raise and to maintain at a constant level the temperature of the preoptic region of a goat for long periods of time This technique will be described in detail later During the entire study the goat was placed in its normal environment (collared in a metabolism cage at a room temperature of 16 to 18 C) Thyroid function was studied using the plasma protein bound I^{131} (PBI^{131}) technique

In the first series of experiments cold stress was induced on 3 consecutive days by the rapid administration of 4 5 *l* of ice water into the rumen On the first day (Fig 1 A) this was done without warming the preoptic region. The

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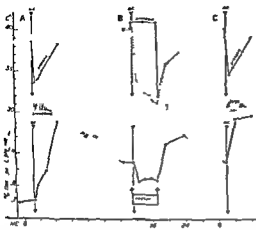

Fig 1

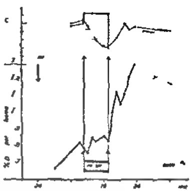

Fig 2

Fig 1 A Thyro d response (plasma PBI) to ruminal cooling (RC)
B Inhibition of this response by preoptic warming (preopt w) to 40 8 C. Note subsequent rise in plasma PBI and onset of shivering when preoptic warming was stopped
C. Ruminal cooling repeated without preoptic warming The dose of carrier free I (60 microcurie) given 3 days prior to the experiment Time of day recorded on abscissa

Fig 2 Diminished release of PBI into plasma during preoptic warming followed by an increased release and a rise of body temperature on cessation of preoptic warming Sixty microcurie carr er free I given at the arrow

temperature of the animal first fell about 5 C but started to rise again within an hour During the fall and the first part of the rising phase of body temperature the animal shivered and the release of thyroid hormone was increased as indicated by a conspicuous rise in plasma PBI¹³¹ The body temperature again reached pre-cooling level after 6 hours However on the second day when ruminal cooling was performed during warming of the preoptic region (to 40 8 C) the reaction of the animal was entirely different (Fig 1 B) The plasma PBI¹³¹ first fell and then stayed at a low level during the 6 hours of preoptic warming Throughout this period shivering was completely suppressed and the body temperature continued to fall (to 31 C) When at this point the preoptic warming was stopped the onset of shivering and a marked rise of plasma PBI¹³¹ occurred To test the reactivity of the pituitary thyroid system, ruminal cooling was again performed without preoptic warming on the third day (Fig 1 C) The response was very much the same as that in the similar experiment on the first day

In another experiment the thyroidal effect of preoptic warming was tested as early as 11 hours after the administration of I¹³¹ i e during the rising phase of plasma PBI¹³¹ (Fig 2) The room temperature was 17 C and no additional cold was applied to the goat The temperature of the preoptic region was raised from 39 7 to 41 2 C and maintained at this level for 6 hours During preoptic warming the plasma PBI¹³¹ ceased to rise and the body temperature

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Blocking of the Thyroid Response to Cold by Local Warming of the Preoptic Region

By

B ANDERSSON, L EKMAN, C C GALE[1] and J W SUNDSTEN[2]

Received 5 June 1962

Local cooling of the preoptic region was recently found to cause a conspicuous activation of the thyroid gland concomitant with the development of marked core hyperthermia (ANDERSSON *et al* 1962 a and b) On the basis of this observation it was postulated that the preoptic "heat loss center (MAGOUN *al* 1938) normally exerts a tonic inhibition of the release of thyrotrophic from the pituitary Due to inactivation of the heat loss center during cold exposure and hypothermia this inhibition would be removed as a link in the metabolic response to cold Further evidence for the assumption would be provided if it could also be shown that local warming of the preoptic region blocks the thyroid response to cold especially since this response has been shown to be mediated via the hypothalamo-pituitary axis (C v EULER and HOLMGREN 1956) The study reported briefly in the following shows that such is really the case

By a special technique of high frequency warming between two bilaterally implanted silver plates, it was possible to raise and to maintain at a constant level the temperature of the preoptic region of a goat for long periods of time This technique will be described in detail later During the entire study the goat was placed in its normal environment (collared in a metabolism cage at a room temperature of 16 to 18 C) Thyroid function was studied using the plasma protein bound I^{131} (PBI^{131}) technique

In the first series of experiments cold stress was induced on 3 consecutive days by the rapid administration of 4 5 *l* of ice water into the rumen. On the first day (Fig 1 A) this was done without warming the preoptic region. The

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Effect of Phlorizin on the in Vitro Release of Histamine from Lung Tissue[1]

By

BERTIL DIAMANT

Received 18 February 1962

Abstract

DIAMANT B *Effect of phlorizin on the in vitro release of histamine from lung tissue* Acta physiol. scand 1962 *56* 97—102 — Histamine release was induced from sensitized guinea pig lung tissue by antigen and from non sensitized rat lung tissue by compound 48/80 Phlorizin (10^{-3} M) had no apparent inhibitory effect on the histamine releasing reactions when elicited under nitrogen in the presence of 5.6×10^{-3} M glucose When the glucose concentration was decreased to 7×10^{-4} M phlorizin (10^{-3} M) inhibited histamine release under nitrogen in both reactions to the same extent as did nitrogenation in the absence of glucose Under nitrogen the enhancing effect of glucose was progressively inhibited by increasing concentrations of phlorizin. In both reactions its inhibitory effect decreased with rising glucose concentration The results are interpreted to indicate that phlorizin inhibits histamine release mainly by inhibiting the transport of glucose

Glucose counteracts the inhibitory effect of nitrogen anoxia on the *in vitro* release of histamine from various animal tissues This has been reported to apply to histamine release induced by *Ascaris* extract (DIAMANT 1960 1961) by compound 48/80 (WESTERHOLM 1960 DIAMANT and UVNÄS 1961 ROTHSCHILD VUGMAN and ROCHA E SILVA 1961) by anaphylatoxin (ROTHSCHILD and BARRETO 1961) and by antigen antibody reaction (MOUSSATCHE and PROVOUST DANON 1961 ROTHSCHILD and BARRETO 1961, DIAMANT 1962 a) In view of these findings the effect of phlorizin on histamine release elicited under nitrogen was investigated Phlorizin is considered to act primarily on the biological transport of glucose an extensive review of its action has been given by LOTSPEICH (1959)

[1] A preliminary report of these results was presented at a meeting of the Swedish Society for Allergy on 30 November 1961 in Stockholm.

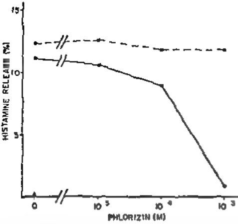

Fig 1

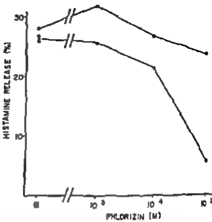

Fig 2

Fig 1 Effect of phlorizin (10^{-5}M — 10^{-3}M) on histamine release under nitrogen from guinea pig lung tissue induced by antigen in the presence of glucose (- - - - = 5.6 × 10^{-3}M; ——— = 7 × 10^{-4} M). *One experiment, each dot represents a single test. Filled triangle =* histamine release under nitrogen without glucose or phlorizin.

Fig 2 Effect of phlorizin (10^{-5}M — 10^{-3}M) on histamine release under nitrogen from rat lung tissue induced by compound 48/80 in the presence of glucose (———• 5.6 × 10^{-3}M; ——— = 7 × 10^{-4} M). Two experiments, each dot represents a single test. Open and filled triangle = histamine release under nitrogen without glucose or phlorizin.

Methods

Histamine release was elicited from sensitized guinea pig lung tissue by antigen (crystallized egg albumin 1 mg/ml) and from non sensitized rat lung tissue by compound 48/80 (35 μg/ml) under the influence of nitrogen, oxygen and glucose as described earlier (DIAMANT and UVNÄS 1961, DIAMANT 1962 a). In all experiments the lung tissue — cut into small pieces — was incubated for 15 min in air; in the relevant experiments phlorizin was present in the incubation medium during this period. Introduction of nitrogen or oxygen followed and, when relevant, addition of glucose. 15 min later the histamine releaser was added. After a further 20 min (during continued nitrogenation or oxygenation) the incubation fluid (phosphate buffer, initial pH 7.1—7.3) was withdrawn. The final pH was determined electrometrically (Beckman pH meter, model G). Histamine was assayed on atropinized guinea pig ileum. The assay was not affected by phlorizin in the concentrations used. The histamine release is given as a percentage of the total histamine content of the lung tissue. All values are corrected for the spontaneous histamine release.

Materials

D dextrose anhydrous (analytical reagent grade, Mallinckrodt Chemical Works, New York, U.S.A.).

Phlorizin (Dr. T. Schuchardt and Co., Munich, Germany).

Crystallized egg albumin (Kebo AB, Stockholm, Sweden).

Oxygen and *nitrogen* (Aga AB, Stockholm, Sweden). The nitrogen contained 0.1—0.3 per cent impurities (part of which consisted of oxygen).

Compound 48/80 (Leo AB, Hälsingborg, Sweden), supplied by the courtesy of Dr. B. Högberg.

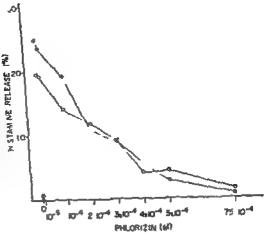

Fig 3 Effect of phlorizin (10^{-5}M — 7.5×10^{-4}M) on histamine release under nitrogen in the presence of 7×10^{-4}M glucose induced by antigen from guinea pig lung tissue (———○) and by compound 48/80 from rat lung tissue (———●) One experiment with each histamine releaser each dot represents a single test Open and filled triangle = histamine release under nitrogen without glucose or phlorizin

Results

Phlorizin in concentrations ranging from 10^{-5}M to 10^{-3}M, had no obvious effect on histamine release elicited under nitrogen in the presence of 5.6×10^{-3}M glucose as shown in Fig 1 (antigen and guinea pig lung tissue) and Fig 2 (compound 48/80 and rat lung tissue) When the glucose concentration was decreased to 7×10^{-4}M, on the other hand 10^{-3}M phlorizin reduced the histamine release in both reactions to almost the same values as those obtained under nitrogen in the absence of glucose

In the presence of oxygen but without glucose 10^{-3}M phlorizin had no inhibitory effect (not shown in Figs 1 and 2) In the presence of oxygen and the absence of glucose and phlorizin 11 per cent of the total histamine content was released by antigen from guinea pig lung tissue Under identical conditions but in the presence of 10^{-3}M phlorizin the value was unchanged The corresponding histamine release from rat lung tissue induced by compound 48/80 amounted to 17 per cent in the absence of phlorizin and to 18 per cent in the presence of 10^{-3}M phlorizin

Under nitrogen the enhancing effect of 7×10^{-4}M glucose on both histamine releasing reactions was progressively lost with rising concentrations of phlorizin (10^{-5}M — 7.5×10^{-4}M) (Fig 3) In both reactions 7.5×10^{-4}M phlorizin completely inhibited the effect of glucose whereas 2.5×10^{-4}M phlorizin produced about 50 per cent inhibition

The inhibitory effect of 2.5×10^{-4}M phlorizin on the histamine release elicited under nitrogen decreased with rising glucose concentration (1.8×10^{-4}M — 2.8×10^{-3}M) as shown in Fig 4 (antigen and guinea pig lung tissue) and Fig 5 (compound 48/80 and rat lung tissue)

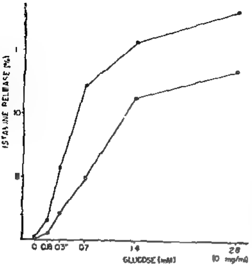

Fig 4 Effect of glucose (1 8 × 10⁻⁴M — 2 8 × 10⁻²M) on histamine release under nitrogen induced by antigen from guinea pig lung tissue in the absence (———•) and in the presence (———•) of phlorizin (2 5 × 10⁻⁴M) One experiment each dot represents a single test

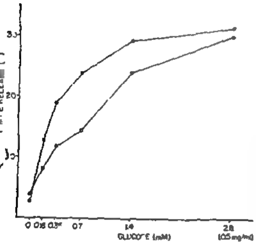

Fig 5 Effect of glucose (1 8 × 10⁻⁴M — 2 8 × 10⁻²M) on histamine release under nitrogen induced by compound 48 80 from rat lung tissue in the absence (———) and in the presence (———•) of phlorizin (2 5 × 10⁻⁴M) One experiment each dot represents a single test.

Discussion

According to LOTSPEICH (1959), "much evidence now points to a membrane site where phlorizin binds a glucose carrier with a high affinity in a number of cell types In his opinion, the inhibitory effect of phlorizin on aerobic utilization of glucose and on oxidative phosphorylation (SHAPIRO 1947, NAGAI 1956 KELLER and LOTSPEICH 1959 a) as well as the finding that phlorizin induces swelling of mitochondria (KELLER and LOTSPEICH 1959 b), appear to be secondary phenomena resulting from phlorizin induced changes in cell and mitochondrial membrane permeability" (LOTSPEICH 1959) In the present investigation 10^{-4}M phlorizin lacked any inhibitory effect on histamine release under oxygen in the absence of glucose an observation which applied to both

antigen induced histamine release from guinea pig lung tissue, and compound 48/80-induced histamine release from rat lung tissue. This finding supports the interpretation of the effect of phlorizin given by LOTSPEICH. Thus, if phlorizin acted primarily on metabolic enzymatic reactions, inhibition of histamine release should occur under the experimental conditions in question.

Under nitrogen in the presence of 5.6×10^{-3} M glucose, 10^{-3} M phlorizin had no apparent effect on the histamine releasing reactions investigated. When the glucose concentration was decreased to 7×10^{-4} M, the histamine release in both reactions was reduced by 10^{-3} M phlorizin to the same values as those found under nitrogen in the absence of glucose. Earlier studies of the effect of phlorizin on anaphylactic histamine release from guinea pig lung tissue were performed in Tyrode solution (which usually contains 5.6×10^{-3} M glucose) and in the presence of air. These factors probably explain why phlorizin was reported to have only a weak inhibitory effect (AUSTEN and BROCKLEHURST 1961) or none (CHAKRAVARTY 1960) in concentrations above 5×10^{-3} M.

Opinions are at variance regarding whether phlorizin competes with glucose for a monosaccharide carrier on the cell membrane in a competitive or a non competitive way. In the kidney of the dog (LOTSPEICH and WORONKOW 1958) and the Ehrlich ascites tumour cells (CRANE, FIELD and CORI 1957) it has been reported to act non competitively. Recently, however, MAKOME and KURIAKI (1961) showed that in the rat intestine phlorizin inhibited glucose absorption competitively. The present investigation concerned the inhibitory effect of phlorizin on histamine release (as related to the enhancing effect of glucose) and not its action on glucose transport, a fact which complicates interpretation of the effect of phlorizin. Despite this, the findings suggest that its mechanism of action is competitive rather than non competitive.

In the present investigation, evidence in favour of the action of phlorizin on the transport mechanism of glucose was obtained by the finding that the glucose concentration of the incubation medium (originally 7×10^{-4} M) decreased less in the presence of phlorizin during incubation than in its absence. This was tested in some experiments (DIAMANT 1962 b) by determining the glucose concentration according to the method of HUGGET and NIXON (1957). It cannot be determined from the present study whether or not phlorizin — in addition to inhibiting glucose transport — affected the histamine releasing reactions in question by inhibiting glycolysis. According to SHAPIRO (1947), however, phlorizin has no such effect on minced cortex of rat kidney. Whatever reactions may be involved, the net effect of phlorizin will be a decreased anaerobic breakdown of glucose and, consequently, a diminished histamine release. The present results therefore support the suggestion that adequate utilization of energy yielding substrates is of significance if the histamine releasing reactions investigated are to proceed.

In the presence of 7×10^{-4} M glucose, almost identical dose response relations were obtained with various concentrations of phlorizin with respect to histamine release under nitrogen induced by antigen from guinea pig lung

tissue, and by compound 48/80 from rat lung tissue This could imply that, in both systems the energy requiring step is equally sensitive to changes in the amount of high-energy compounds available Moreover it argues in favour of the possibility that the energy requiring step is common to both reactions

This investigation has been supported by grants from Arbetscirkeln Diabetika and from Karolinska Institutet Sweden and from the National Institutes of Health (A 4064 C 1) U S A. for which grateful acknowledgement is made

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Acta physiol. scand. 1962. 56. 103—111

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Comparison Between the Effects of Glucose and Sodium Succinate on the in Vitro Release of Histamine from Guinea-Pig and Rat Lung Tissue[1]

By

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Received 16 February 1962

Abstract

DIAMANT, B. *Comparison between the effects of glucose and sodium succinate on the in vitro release of histamine from guinea-pig and rat lung tissue.* Acta physiol. scand. 1962. *56.* 103—111. — Histamine release elicited under nitrogen anoxia by antigen from sensitized guinea pig lung tissue and by compound 48/80 from non sensitized rat lung tissue was enhanced by the presence of glucose but not by that of sodium succinate. In the presence of oxygen, sodium succinate as well as glucose enhanced antigen induced histamine release from guinea pig lung tissue. In contrast, histamine release from rat lung tissue induced by antigen, as well as by compound 48/80, was not enhanced by sodium succinate in the presence of oxygen, whereas glucose was effective in both reactions. Possible reasons for this species difference are discussed in the light of the supposition that the histamine releasing reactions investigated depend on adequate utilization of energy yielding substrates and of high-energy compounds.

The enhancing effect of glucose on various *in vitro* histamine releasing reactions inhibited by oxygen lack has been reported in several investigations (for references, see Table I). Among these reactions are anaphylactic histamine release from rat and guinea pig lung tissue, and compound 48/80-induced histamine release from rat lung tissue. In the presence of oxygen, the two latter reactions were also found to be enhanced by glucose (Table I).

[1] A preliminary report of these results was presented at a meeting of the Swedish Society for Allergy on 30 November 1961 in Stockholm.

Table I *Effects of glucose and succinate on histamine release in vitro studied under nitrogen and oxygen as reported in earlier investigations*

Reference	Histamine releaser	Species, Tissue	Effect of
			Glucose in nitrogenated medium
DIAMANT (1960, 1961)	*Ascaris* extract	Rat lung	–
WESTERHOLM (1960)	Compound 48/80	Cat skin	–
DIAMANT and LYNAS (1961)	Compound 48/80	Rat lung	–
ROTHSCHILD *et al.* (1961)	Compound 48/80	Rat diaphragm	–
MOUSSATCHÉ and PROVOOST DANON (1961)			
DIAMANT (1962 a)	Antigen	Rat lung	–
MOUSSATCHÉ and PROVOOST DANON (1961)			
ROTHSCHILD and BARRETO (1961)			
DIAMANT (1962 a)	Antigen	Guinea-pig lung	+
ROTHSCHILD and BARRETO (1961)	Anaphylatoxin	Guinea-pig lung	–
			Succinate in nitrogenated medium
YAMASAKI *et al.* (1960)	Antigen	Guinea pig lung	0
			Glucose in oxygenated medium
DIAMANT (1960, 1961)	*Ascaris* extract	Rat lung	+
WESTERHOLM (1960)	Compound 48/80	Cat skin	0
DIAMANT and LYNAS (1961)	Compound 48/80	Rat lung	+
ROTHSCHILD *et al.* (1961)[1]	Compound 48/80	Rat diaphragm	+
YAMASAKI *et al.* (1960)			
DIAMANT (1962 a)	Antigen	Guinea-pig lung	+
			Succinate in oxygenated medium
MOUSSATCHÉ and PROVOOST DANON (1957)			
YAMASAKI *et al.* (1960)	Compound 48/80	Guinea-pig lung	0
MOUSSATCHÉ and PROVOOST DANON (1957)			
YAMASAKI *et al.* (1960)			
AUSTEN and BROCKLEHURST (1961)			
CHAKRAVARTY (1961)	Antigen	Guinea-pig lung	+
YAMASAKI *et al.*	Decylamine	Guinea-pig lung	0

[1] = experiments performed in air – = enhancing effect. 0 = no effect.

Among the intermediates of the tricarboxylic acid cycle succinate in particular has been shown to increase anaphylactic histamine release as well as oxygen consumption, in guinea pig lung tissue (Table I). It did not, on the

contrary, have any enhancing effect on histamine release from guinea pig lung tissue when induced by compound 48/80 although the oxygen consumption was increased These observations were put forward as evidence of a difference between the mechanism of action in histamine release by antigen and by chemical histamine releasers (MOUSSATCHÉ and PROVOUST DANON 1957, YAMASAKI MURAOKA and ENDO 1960) In the guinea pig however compound 48/80 is generally considered to act non enzymatically (HOGBERG and UVNAS 1957 BORELS 1960 a) whereas anaphylactic histamine release is usually believed to involve enzymatic reactions

An account is given in the present paper of a reinvestigation of the enhancing effect of sodium succinate on histamine releasing reactions known to involve enzymatic processes as judged by their activation by glucose

Methods

Histamine release was induced by antigen (crystallized egg albumin 1 mg/ml) from sensitized guinea pig and rat lung tissue and by compound 48/80 (35 μg/ml) from non sensitized rat lung tissue using methods described elsewhere (DIAMANT and UVNAS 1961 DIAMANT 1962 a) In all experiments the lung tissue — cut into small pieces — was incubated for 15 min prior to addition of the histamine releasing agent. The incubation fluid (phosphate buffer initial pH 7 1—7 3) was withdrawn 20 min later and the final pH determined electrometrically (Beckman pH meter model G) Glucose and/or sodium succinate was present from the beginning of incubation During the whole incubation period the lung tissue was exposed to oxygen or nitrogen Histamine was assayed on atropinized guinea pig ileum Sodium succinate (in the concentration used) did not affect the assay The histamine release is given as a percentage of the total histamine content of the lung tissue All values are corrected for the spontaneous histamine release Each type of experiment was performed 2 to 4 times mean and individual values of duplicate samples in one representative experiment are shown

Materials

D dextrose anhydrous (analytical reagent grade Mallinckrodt Chemical Works, New York, U S A)

Sodium succinate (Hopkin and Williams Ltd. Essex England)

Crystallized egg albumin (Kebo AB Stockholm, Sweden)

Oxygen and *nitrogen* (Aga AB Stockholm Sweden) The nitrogen contained 0 1—0 3 per cent impurities (part of which consisted of oxygen)

Compound 48/80 (Leo AB Halsingborg Sweden) supplied by the courtesy of Dr B Hogberg

Results

Fig 1 shows the effect of equimolar concentrations (5 6 × 10^{-3}M) of glucose and sodium succinate on histamine release under nitrogen elicited by antigen from guinea pig lung tissue and by compound 48 80 from rat lung tissue In the absence of glucose as well as of sodium succinate no histamine release was induced by antigen from guinea pig lung tissue on an average in the presence of glucose 17 per cent of the total histamine was released in the presence

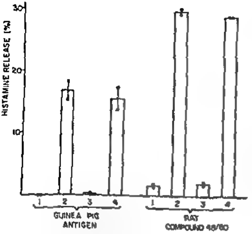

Fig 1 Effect of glucose (6 × 10^{-3} M) and sodium succinate (5 6 × 10^{-3} M) on histamine release under nitrogen induced by antigen from guinea pig lung tissue and by compound 48/80 from rat lung tissue Mean and individual values of duplicate samples in *one* experiment with each histamine releaser are shown
1 No glucose or sodium succinate
2 Glucose
3 Sodium succinate
4 Glucose + sodium succinate

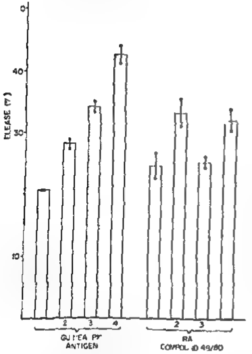

Fig 2 Effect of glucose (5 6 × 10^{-3} M) and sodium succinate (5 6 × 10^{-3} M) on histamine release under oxygen induced by antigen from guinea pig lung tissue and by compound 48/80 from rat lung tissue Mean and individual values of duplicate samples in *one* experiment with each histamine releaser are shown
1 No glucose or sodium succinate
2 Glucose
3 Sodium succinate
4 Glucose + sodium succinate

of sodium succinate less than 1 per cent and in the presence of both glucose and sodium succinate 16 per cent Corresponding values for histamine release induced by compound 48/80 from rat lung tissue amounted to 2 30 2 and 29 per cent Under anoxic conditions sodium succinate evidently neither enhanced histamine release nor influenced the enhancing effect of glucose in the reactions investigated

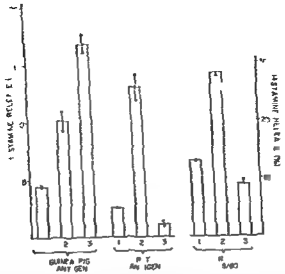

Fig 3 Effect of glucose (5 6 × 10⁻³M) and sodium succinate (5 6 × 10⁻³M) on histamine release under oxygen induced by antigen from guinea pig lung tissue and by antigen and compound 48/80 from rat lung tissue Mean and individual values of duplicate samples in *one* experiment on each histamine releasing reaction are shown The lung tissue was pre-exposed to nitrogen for 35 min (without glucose or sodium succinate) and reincubated under oxygen after washing
1 No glucose or sodium succinate
2 Glucose
3 Sodium succinate

The experiments shown in Fig 2 were performed in the same way as those in Fig 1 except that nitrogen was replaced by oxygen Anaphylactic histamine release from guinea pig lung tissue averaged 21 per cent in the absence of glucose and sodium succinate In the presence of glucose ($5\ 6 \times 10^{-3}$M) 28 per cent of the total histamine content was released in the presence of sodium succinate ($5\ 6 \times 10^{-3}$M) 34 per cent and in the presence of both glucose and sodium succinate 43 per cent The corresponding histamine release elicited by compound 48/80 from rat lung tissue amounted to 25 33 25 and 32 per cent respectively Thus under oxygen the anaphylactic histamine release from guinea pig lung tissue was enhanced to a greater degree by sodium succinate than by glucose optimal activation being obtained when glucose and sodium succinate were present concurrently in the medium Compound 48/80-induced histamine release from rat lung tissue was on the contrary enhanced only by glucose and was uninfluenced by the presence of sodium succinate

A smaller difference than that shown in Fig 2 was sometimes observed between the histamine release elicited under oxygen from rat lung tissue by compound 48/80 in the presence of glucose and in its absence It was shown in an earlier study that when rat and guinea pig lung tissue were pre incubated under nitrogen in the absence of glucose for 35 min,

release under oxygen in the absence of glucose diminished whereas under oxygen in the presence of glucose only slight inhibitory effects were noted (DIAMANT 1962 b) To obtain a larger difference between the histamine release under oxygen in the presence and absence of glucose which would permit better evaluation of the effect of sodium succinate rat and guinea pig lung tissue were pre incubated under nitrogen for 35 min without glucose or sodium succinate After washing with substrate free phosphate medium the tissue was divided, and reincubated under oxygen with the usual procedure Three experiments of this kind are shown in Fig 3 When guinea pig lung tissue was exposed to antigen under oxygen in the absence of glucose and sodium succinate 4 per cent of the total histamine content was released The value in the presence of 5.6×10^{-3}M glucose was 10 per cent and in the presence of 5.6×10^{-3}M sodium succinate 17 per cent In rat lung tissue, the corresponding figures were 3, 13 and 1 per cent after exposure to antigen and 13 28 and 9 per cent after exposure to compound 48/80 It is evident that the histamine release was enhanced by glucose in all these reactions Sodium succinate had a greater activating effect than glucose in anaphylactic release from guinea pig lung tissue, whereas it failed to enhance the release from rat lung tissue when elicited by antigen as well as by compound 48/80

Glucose was found to decrease the final pH of the incubation medium by about 0.2 unit in all experiments indicating an accumulation of lactic acid No change in the final pH was on the contrary, produced by sodium succinate any of the experiments

Discussion

The present investigation supports the view that the histamine releasing reactions investigated involve some energy requiring step Theoretically lack of high energy compounds and consequently inhibition of histamine release can be induced in several ways These problems were discussed in a recent paper in the light of the effect of pre-exposure of rat and guinea pig lung tissue to anoxic glucose free conditions prior to exposure to compound 48/80 *Ascaris* extract and antigen under various metabolic situations with oxygen nitrogen and glucose (DIAMANT 1962 b) It was suggested that adequate utilization of energy yielding substrates and of high energy compounds may be of significance for the histamine releasing reactions in question This suggestion is borne out by the present investigation

Additional evidence was presented by MOUSSATCHÉ and PROVOUST DANON (1961) They showed that the enhancing effect of glucose on antigen induced histamine release from guinea pig lung tissue elicited under nitrogen was correlated to the glycolytic activity of the tissue (as judged by the increased Q_{CO} induced by glucose) They further observed that in the presence of mono-iodoacetate, glucose enhanced neither Q_{CO} nor anaphylactic histamine release

YAMASAKI MURAOKA and ENDO (1960) stated that under nitrogen anoxia sodium succinate lacked any enhancing effect on anaphylactic histamine release from guinea pig lung tissue This was confirmed in the present investigation and was also shown to apply to histamine release elicited by compound 48/80 from rat lung tissue In these experiments the enhancing effect of glucose especially when combined with sodium succinate served as a check that histamine release could in fact take place under the anoxic experimental conditions used It can therefore be concluded that sodium succinate has no effect on histamine release under conditions where it cannot be metabolized

YAMASAKI *et al* (1960) further noted that in the presence of oxygen anaphylactic histamine release from guinea pig lung tissue was increased more by sodium succinate than by glucose Moreover, in the presence of both sodium succinate and glucose the increase was greater than that produced by sodium succinate alone The present study confirms these results with respect to anaphylactic histamine release from guinea pig lung tissue Histamine release from rat lung tissue — induced by antigen as well as by compound 48/80 — was not, on the contrary found to be enhanced by sodium succinate in the presence of oxygen This finding lends support to CHAKRAVARTY's (1960) suggestion *i e* that a species difference exists between the rat and guinea pig with respect to histamine releasing reactions from lung tissue

The reason for the species difference noted in the present investigation is so far unknown In the presence of sodium succinate the oxygen consumption of guinea pig lung tissue increases (MOUSSATCHE and PROVOUST DANON 1958 YAMASAKI *et al* 1960 CHAKRAVARTY 1961) In rat lung tissue a similar increase in oxygen consumption was observed in the presence of sodium succinate (DIAMANT and FREDHOLM 1962) These observations indicate that in the lung tissue of both species oxidation of sodium succinate occurs with a resulting formation of high energy compounds This is borne out by the finding of LEE YU and BURSTEIN (1960) *i e* that sodium succinate (as well as glucose) besides increasing the oxygen consumption also increased the adenosine triphosphate (ATP) and phosphocreatine content of cat papillary muscle Consequently the species difference noted in regard to the enhancing effect of sodium succinate on histamine release cannot be ascribed to sodium succinate not being metabolized in rat lung tissue

In previous communications from this laboratory (see HOGBERG and UVNAS 1957 1960 BOREUS and CHAKRAVARTY 1960 BOREUS 1960 b UVNAS and THON 1961 UVNAS 1961 DIAMANT and UVNAS 1961) evidence has been presented showing that the histamine release from tissues — induced by antigen antibody reaction and by chemical (compound 48/80) and biologically occurring histamine releasers (*Ascaris Cyanea*) — is the result of enzymatic reactions taking place in the mast cells which thereby undergo morphological changes The histamine release from various tissues and from isolated rat mast cells as well as the morphological changes in mast cells *in situ* caused by these agents

are, on the whole similarly affected by factors influencing metabolism This has led UVNAS (1961) to conclude that the mast cell is the site of the metabolic processes necessary for the release of histamine'

The inability of sodium succinate to enhance histamine release from rat lung tissue noted in the present investigation could therefore be explained on the following assumptions Rat mast cells — in contrast to those of the guinea pig — might lack the prerequisites either for uptake of sodium succinate from the incubation medium or for its utilization in energy generating reactions Another reason might be that *in rat mast cells the postulated energy requiring step in* the reactions leading to histamine release cannot utilize energy (ATP) generated in mitochondria through the oxidation of sodium succinate Although at present, this is only hypothesis it opens up interesting aspects for future work

In those investigations dealing with histamine release as correlated to metabolic reactions in minced tissues, it has generally been assumed that the oxygen consumption of the tissues reflects the metabolic reactions of the mast cells The present finding that sodium succinate does not stimulate histamine release from rat lung tissue despite the fact that the oxygen consumption is increased (DIAMANT and LRIDHOLM 1962) emphasizes the possibility that such an assumption may not be valid Only a negligible part of the total metabolism registered could probably be accounted for by the mast cells of the tissues It is therefore suggested that before any conclusions are drawn from discrepancies between histamine release and oxygen consumption of whole tissues the results should be confirmed on isolated mast cells with special regard to the content and utilization of ATP in these cells at the time of exposure to histamine releasing agents

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A Method for Study of the Interrelation Between EEG and Blood-Brain Barrier Phenomena

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Abstract

Flodmark, S and O Steinwall. *A method for study of the interrelation between EEG and blood brain barrier phenomena* Acta physiol scand 1962 *56* 112—119 — An experimental procedure has been elaborated to facilitate selective effects exerted by agents applied within the cerebral vessels on EEG and blood brain barrier phenomena In rabbits short term (less than 1 minute) perfusion of one hemisphere is performed via the ipsilateral internal carotid artery with a pressure adjusted so as to obtain displacement of the blood This displacement is controlled by inspection of the pial vessels through a trephine opening The technique implies control of the active concentration *in loco* of the applied agents and of the application time hence graded influences near the threshold levels can be obtained

Structural and metabolic considerations make it conceivable that low grade damage of the blood brain barrier may be produced with no significant effect on the neuronal activity as reflected in EEG (except for the brief influence from the blood deprivation) After such damage intravenous administration of suitable substances unable to pass the intact barrier may give rise to unilateral EEG changes and thus reveal the defective barrier function Two model experiments are reported The first one shows that unilateral blood deprivation *per se* (for about 1 min) exerts a marked but reversible effect on the EEG without damage to the blood brain barrier as tested by intravenously injected trypan blue The second experiment illustrates an attempt to induce barrier damage without significant changes in the EEG

The electrical activity as observed in the EEG is essentially generated in neuronal structures and thus primarily reflects the state of the neurons. It has not been shown that the glia cells actually produce electrical phenomena of direct significance for the EEG (see TASAKI and CHANG (1958)), but they no doubt exert an indirect effect on the EEG by virtue of their decisive influence on neuronal metabolism (i. a. see HYDÉN (1960)). The interposition of glial elements between neurons and brain capillaries strongly suggests that the perivascular glia is involved in the regulation of blood brain exchange of substances. This does not necessarily mean that the special phenomena generally accorded to the blood brain barrier concept are solely glial effects. This concept probably encompasses a multiplicity of mechanisms the background of which may be found in different structures and metabolic factors (i. a. see BROMAN (1955), DOBBING (1961), EDSTRÖM and STEINWALL (1961), TSCHIRGI (1961)).

Correlations between the EEG and blood brain barrier phenomena have been reported by many investigators (i. a. PRADOS *et al.* (1945), AIRD (1949), BLOOR *et al.* (1951 b), FUNDERBURK and CASE (1951), GONSETTE (1956), PURPURA *et al.* (1958), PURPURA and CARMICHAEL (1960)). The present technique for experiments on rabbits has been elaborated in order to penetrate the possibilities of inducing differentiated effects on neuronal activity and barrier functions.

During EEG recording various agents are applied within the blood vessels of one hemisphere, the other serves as a control with respect to EEG as well as to the influence of the applied agents on barrier function. Special attention has been directed to the achievement of a reliable control of the concentration *in loco* of the applied agents as well as of the application time. By that means influences near the threshold level can be obtained and this presumably favours the occurrence of dissociated influences on either the barrier or the EEG. In the present paper the experimental procedures and two illustrative experiments will be reported and discussed.

Experimental procedures

Adult rabbits in urethane anaesthesia were used. Blood pressure was measured by means of a mercury manometer connected to a femoral artery. In some experiments the electrocardiogram and the respiratory rate were simultaneously recorded.

EEG recording. After preliminary trials with different types of electrodes chloridized silver screws (10 mm in length) were found most suitable. The screws were applicated intraosseally without injury to the lamina interna of the bone, because pressure lesions easily occur at epidural application even when the dura is intact. Further fixation and isolation of the electrodes was obtained with plastic cement. The electrodes were symmetrically placed over the hemispheres, anteriorly over the motor region just in front of the sutura coronaria, and posteriorly over the occipital region. The distance from the midline was about 5 mm. Bipolar recording with an eight-channel Grass EEG machine was used.

Intravasal application of agents The application of agents within the vessels of one hemisphere was performed according to a technique developed by BROMAN and OLSSON (1948 1956) and modified by STEINWALL (1958) The solution was injected through a plastic catheter secured in one common carotid artery after ligation of its proximal part and its external branches The injection pressure was kept at the blood pressure level so as to obtain displacement of the blood from the ipsilateral hemisphere This was controlled by inspecting the pial vessels through a trephine opening with the transparent dura left intact The injection time was kept within 30—45 sec Afterwards blood from the other arteries supplying the circle of Willis recirculated through the perfused vessels In the event that highly toxic agents be employed a reduction of their concentration in the general circulation might be attained by drainage from the external jugular vein during the injection

The applied chemical solutions were approximately neutral and isotonic and were filtered before injection When the application time is kept within the narrow limits mentioned the concentration of the noxious agents primarily determines their effect whereas the injected volumes (usually between 5 and 10 ml) are of minor importance.

Testing the state of the blood brain barrier In order to ascertain whether or not the intravasal application of the agents induced inhibition of the blood brain barrier suitable indicator substances which are unable to pass from blood to brain under normal barrier conditions were introduced into the general circulation In the current experiments the indicators were chosen from among the group of anion dyes and other organic acids discussed by STEINWALL (1961) The effect on the EEG was studied as a functional indication of the state of the barrier Furthermore the brains were scrutinized *post mortem* to reveal any staining by the intravitally applied indicator dyes The animals were killed by exsanguination and the cerebral vessels rinsed with saline under one meter of pressure for one minute before removal of the brain

Comment The described procedure for intravasal application of solutions within one hemisphere involves an obvious interference with the cerebral blood supply by the occlusion of one carotid artery and the brief blood deprivation during the injection

Occlusion of one carotid artery in rabbits brings about circ effects which have been thoroughly studied see McDONALD and), J
and OLIN (1960) KRUPP (1961) Through the well
the ipsilateral hemisphere is sufficiently supplied
systematic blood pressure may increase up to 20
occlusion of one carotid artery induces only
type in the EEG In the present series this
of both carotid arteries or unilateral occlusio
blood pressure by about 50 per cent (by me
no significant effect on the EEG in our

The deprivation of blood from one hemisph
obviously gives rise to anoxic effects calling
influence on the EEG By iterated controls
been found that such blood deprivation by
glucose uniformly induces unilateral depres
of the frequency pattern after 20 to 30

more than 60 seconds the EEG changes disappear within half a minute after the injection was terminated These transient effects thus need not disturb the interpretation of the more protracted changes derived from chemical influences when certain noxious agents are injected

With a technique similar to the present one BLOOR *et al* (1951 a 1951 b) studied EEG and blood brain barrier effects however without control of the blood expulsion which with our method is checked by inspection through the trephine opening In other methods of principally the same type stress has been laid upon minimizing interference with cerebral circulation. To that end the agents have been injected directly in the streaming blood of one carotid artery by means of a very thin needle (SHIMIZU *et al* 1952) or introduced via a catheter secured in a branch opening into the carotid trunk (McDONALD and POTTER 1951 ROTHBALLER and JARVIK 1958 ROVIT *et al* 1960) All these methods obviously permit distinct unilateral influences but they are not designed to yield control as to the concentration of the applied agents *in loco*

Urethane anesthesia has been employed in most experiments This drug was chosen because it induces a continuous anesthesia at a level where alerting effects may easily be obtained by external stimuli These variations in alertness often enhance the asymmetric EEG changes sought. In our opinion this type of anesthesia has no inherent draw backs for the current experiments

Model experiments

I *Effects of unilateral blood depri ation*

An adult rabbit weighing 2 2 kg in urethane anesthesia was used Registra tion of the blood pressure reaction at temporary clamping of the carotid arteries showed an elevation of 5—10 mm from 90 mm Hg

Symmetric EEG records were obtained before and after catheterization of the left carotid artery Ringer s solution (with glucose to 0 25 %) was injected twice through the carotid catheter causing blood expulsion from the left hemisphere The first injection (20 ml) lasted for 55 sec In the EEG depressed amplitudes and an increase of slow waves appeared 20 sec after the start of the injection These changes were limited to the left side and disappeared promptly when the injection ceased Ten minutes later a second injection (40 ml) was administered for a period of 2 min (Fig 1 A B) About half a minute after the start the same type of left sided EEG changes commenced and gradually increased giving rise to a pronounced asymmetry with very slow waves (1—2 c s) while the right side showed arousal effects At termination of the injection distinct changes on the left side persisted for about one minute and a slight asymmetry was visible for at least another minute An i v injection of 18 ml trypan blue (1 %) was started 20 sec after the end of the carotid injection (Fig 1 C The EEG during the remaining 15 min of the experiment was symmetric and without any noteworthy features (Fig 1 D) After exsanguination and

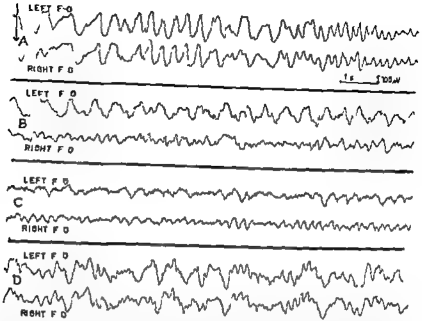

Fig 1 *EEG effects induced by blood deprivation from the left hemisphere (urethane anesthesia)* A Symmetric record with alerting effect obtained at the start of injection (see arrow) of Ringer's solution via the left internal carotid artery B Delta activity over the left hemisphere after blood deprivation for 80 sec. C Persisting left-sided EEG changes 2 min after injection during i v administration of trypan blue D Symmetrical EEG pattern obtained at the end of the experiment (about 20 min) (F O bipolar fronto-occipital leads)

... of the cerebral vessels the brain was removed No staining of either hemisphere was observed

Comment This type of experiment demonstrates how unilateral ischemia affects the EEG without simultaneous influence on the blood brain barrier to acid dyes In addition to such analysis of ischemia effects *per se* it is possible to study how one hemisphere previously subjected to ischemia of varying duration reacts to certain intravenously given agents *e g* analeptic drugs in comparison with the other hemisphere

2 *Effects induced by the organic acid Urokon*©

A rabbit weighing 1 8 kg anesthetized with urethane showed a symmetric EEG (Fig 2 A) before application of Urokon (sodium acetrizoate) Via a carotid catheter 14 % Urokon (6 ml) was perfused through the left hemisphere for 45 sec The EEG recording starting 4 min after this injection showed essentially the same symmetric pattern as before (Fig 2 B) Ten minutes after the carotid injection the general circulation was loaded with Urokon by i v injection of 12 ml 40 % solution A few minutes later the EEG showed a marked asymmetry with dominance of slow waves on the left side especially after arousal stimulations About 10 min after the i v injection frequent spikes with

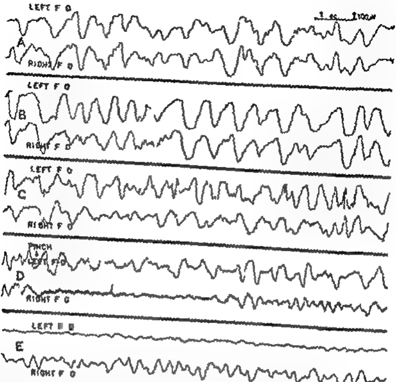

Fig 2 EEG changes after unilateral application of Urokon (by left sided carotid injection) and after subsequent intravenous loading with Urokon A Control record (urethane anesthesia) B Four minutes after left-sided carotid injection of 14 % Urokon (6 ml) for 45 sec Essentially the same symmetric pattern as in A. C Spike potentials on the left side starting about 10 min after i.v administration of 12 ml 40 % Urokon D EEG recorded 2 hours later showing symmetric resting EEG but asymmetric arousal effects E Flat line on the left side about 15 min after the second i v injection of Urokon (40 % 15 ml) (F O bipolar fronto-occipital leads)

high amplitudes were observed on the same side (Fig 2 C.) The abnormalities vanished within an hour but alerting stimuli still evoked transient asymmetric response (Fig 2 D) A second i.v. injection of Urokon (15 ml 40 %) was performed about two hours after the first About 15 min later a marked depression of the amplitudes practically down to flat lines occurred on the left side, while no significant influence could be seen on the right side (Fig 2 E) As intravital dye indicator 40 ml of trypan blue (0.5 %) was given i.v. in two portions before and after the second Urokon administration The rinsed brain showed a moderate degree of staining by trypan blue limited to the left hemisphere

Comment From earlier investigations the X ray contrast medium Urokon (sodium acetrizoate) is known to cause barrier damage (BROMAN and OLSSON 1948 and 1956 BLOOR *et al* 1951 a) When injected via the carotid artery in the concentration and with the technique used here this damage could be

expected to be of a low degree (STEINWALL 1958) and primarily affect the structures responsible for the barrier function This supposition is in agreement with the observation that the EEG in this phase of the experiment did not show any significant changes Afterwards when a large amount of Urokon was administered i v this polar compound could invade the hemisphere with the defective barrier and cause marked influence of the EEG on this side Thus Urokon in this experiment played a double role as local barrier damaging agent and as indicator of the barrier defect In either or both of these roles Urokon may be replaced by others of a large group of organic anions including many dyes (STEINWALL 1961)

Analogous studies on organic cation compounds are in progress at our laboratory with the purpose of supplementing the information on selective barrier inhibition (STEINWALL in press) as well as developing clinical methods for testing damage to the blood brain barrier (FLODMARK 1958 1962)

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Later the value of the rectal temperature as index of the regulated body temperature has been doubted (cf reviews by GRANT 1951 and HARDY 1961) MEAD and BONMARITO (1949) for instance found that the rectal temperature measured simultanously at different depths under various conditions of warming or cooling of the body showed considerable differences The lowest temperature was found at a depth of 20 cm and the authors suggested that this was due to a local cooling caused by venous blood coming from the skin of the lower extremities

In the studies on temperature regulation during exercise (NIELSEN 1938) the rectal temperature was measured at great depth (20—25 cm) In the present investigation it was therefore planned to study the rectal temperature at different depths during work at different environmental temperatures and to compare the results with the temperature measured in the lower oesophagus This temperature was considered by GERBRANDY SNELL and CRANSTON (1954) to reflect the temperature changes at the central temperature receptors COOPER and KENYON (1957) found that the deep oesophageal temperature was very close to the temperature of the aorta and CARLSTEN and GRIMBY (1957) that it was a good index of the heart blood temperature

Methods and Procedure

The rectal temperature was measured at four depths (12 cm 17 cm 22 cm and 27 cm from the external anal sphincter) by four copper constanten thermocouples mounted in a 6 mm synthetic rubber tube The 27 cm thermocouple was soldered to a silver knob (8 mm in diameter) which protruded 4 mm from the tip of the tube and the other three thermocouples to 3 silver cylinders (8 mm in diameter) which were exposed through the surface of the rubber tube as 4 mm wide rings The copper wire consisted of 10 × 0 1 mm strands The stranded constantan wire (8 × 0 18 mm) served as a common lead for all four thermocouples and was connected to a common reference thermocouple from which the stranded copper wire led to a selector switch

The possibility of an error due to heat conduction along the wires between neighbouring thermocouples was tested by placing the thermocouples one in each cell of a waterfilled box The cells were separated from one another by insulating partitions and the water was kept thoroughly mixed The test showed that lowering or elevating the temperature in one cell 0 5 to 1 C influenced the measurement from the thermocouple in the neighbouring cell by only a few hundredths of a degree centigrade

The oesophageal temperature was measured by a copper-constantan thermocouple soldered to a 4 3 mm silver knob at the tip of a 4 mm rubber tube This thermocouple was inserted through the nose and swallowed to a depth of about two cm above the diaphragm i e a distance of 45—50 cm from the nostril The exact distance was in each subject determined by means of a roentgenological examination In some of the experiments the sublingual temperature was measured by a similar thermocouple The reference thermocouples were tied to the bulb of a mercury thermometer which was kept at 37 C in a thermos bottle and read at each measurement The thermocurrent was read on a Cambridge Spot Galvanometer placed on a vibration free support. The calibration was performed in a stirred thermoregulated water bath with each single thermocouple being fastened to the bulb of the mercury thermometer The

calibration was done at 5 C, 20 C and 30 C and was frequently checked The measurements of the body temperature are considered to be accurate to about ± 0 0°5 C.

The work was performed on a KROGH bicycle ergometer in which the saddle had been replaced by an armchair In experiments in which the work was done with the arms the subject sat on a high stool and the pedals were replaced by handles The respiratory metabolism from which the heat production was calculated was determined by the DOUGLAS-bag method.

In some of the experiments the subject breathed air saturated with water vapor at body (deep oesophageal) temperature In these experiments the subject inspired from an aluminium cylinder (40 cm long and 25 cm in diameter) The steam was led from a kettle of boiling water through two nylon tubes to the cylinder in which an arrangement of screens insured a thorough mixing of the water vapor and the inspired air Coarse adjustment of the inspired air to body temperature could be obtained by regulating the heat supply to the boiling water while precise adjustment was obtained by an electric fan which was started (either manually or automatically from a thermistor placed in the air stream) when the temperature rose above the desired value It was not possible to regulate the air temperature when using the ordinary respiration valves of metal but low heat capacity valves made of plastic proved to be applicable In order to establish the proper temperature gradient from the saturation cylinder to the mouthpiece, before the subject was connected an airstream (7 liters per minute) was provided by means of a water suction pump

The experiments were carried out in a climatic chamber in which the temperature even during heavy muscular exercise could be maintained at any desired level higher than about 5 C The relative humidity was determined by an aspiration psychrometer and found to average about 70 % at 5 C, 50 % at 20 C and 25 % at 30 C corresponding to vapor pressures of respectively 4 6 7 8 and 7 9 mm Hg At these vapor pressures an adequate evaporation of the produced sweat was obtained in all experiments except in those performed at 30 C In the experiments done at high environmental temperature an fan was used to produce an air movement of 0 2—0 3 m/sec around the subjects and about 0 5—1 0 m/sec at chest height At 30 C forced air movement during work influences the calorie demand of the environment only slightly as indicated by the small effect on the heat loss by radiation + convection from the subject (NIELSEN 1938)

The subjects who were young male students (age 19—24 years) arrived at the laboratory in the morning and sat for 15 to 30 minutes in the ergometer chair with the thermocouples in place before the start of the work experiment.

Results

Fig 1 shows rectal and oesophageal temperatures in experiments with a work intensity of 900 mkg/min performed at the three environmetal temperatures 5 C 20 C and 30 C on subject T N The rectal temperature was measured at 12 17 22 and 27 cm depth and the oesophageal temperature just above the diaphragm The points on the curves represent in all three figures the averages from 4 exp It is seen that at this work intensity there are only small and unsystematic differences between the rectal temperatures at the four depths. This can also be seen from Table I which contains the results from the "steady state (30 to 60 min after the start of work) at 900 mkg/min with 4 subjects Table II which presents the mean differences between the

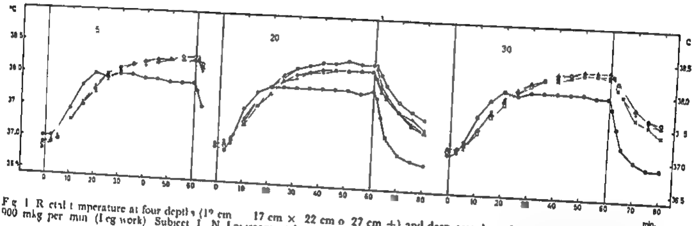


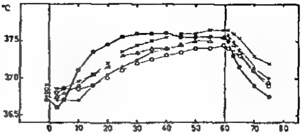
Fig 1 Rectal temperature at four depths (12 cm · 17 cm × 22 cm o 27 cm +) and deep oesophageal temperature ● Work intensity 900 mkg per min (leg work) Subject I N Environmental temperature 5 C 10 C and 30 C Average of results from 4 experiments

Table I Rectal temperature and d ep oesophagus temperature from steady state of work (a erages of measurements from 50 to 60 min after start of work)

	Subject	Room temp C	Heat pro-duc tion Cal/min	Num ber of exp	T_R t. C 12 cm	17 cm	22 cm	27 cm	Mean T_{Rect} C	$T_{Oes.}$ C	$T_{Rect.}-T_{Oes.}$ C
Work with the legs 900 mkg/min.	J R	5	8.84	5	38 16	38.12	38 12	38 16	*38 14*	*37.91*	0.[illegible]3
		20	9 00	7	38 12	38 05	38 06	38 10	*38 08*	*37.8[illegible]*	0.23
		[illegible]0	8 25	5	38 15	38 08	38 13	38 06	*38 11*	*37.84*	0.27
									38 11*	37 86	
	E A	5	7 90	4	38 37	38.24	38 37	38 34	*38.33*	*38 05*	0 [illegible]8
		20	7 90	5	38 41	38.19	38 41	38 27	*38.3[illegible]*	*37.98*	0 34
		30	7 9[illegible]	4	38 34	38 06	38.23	38.14	*[illegible] 19*	*37 86*	0 33
									38 28*	37 96	
	T N	5	7 79	4	38 26	38 25	38 29	38.[illegible]6	*[illegible]8.27*	*37.9[illegible]*	0 3[illegible]
		20	7 64	4	38 23	38 21	38.34	38 34	*38 28*	*37 89*	0 39
		30	7 74	4	38 36	38.29	38 32	38 32	*38.3[illegible]*	*37.97*	0.3[illegible]
									38.29*	37 93	
	C H	5	—	5	38 07	38 08	38 03	38 07	*38 06*	*37 80*	0.[illegible]
		20	—	5	37 98	38 04	38 02	37 93	*37.99*	*37 71*	0.28
		30	—	5	37 96	37 98	37 98	37 94	*37.97*	*37 63*	0.34
									[illegible] 01*	37 71*	
do Insp airsat with water vapor at body temp	T N	20	7 64	4	38 70	38 63	38 67	38 64	*38 66*	*38 19*	0 47
	E A	20	—	2	38 09	38.32	38 26	38 19	*[illegible]8 2*	*37 75*	0.47
Work with the arms 450 mkg/min	T N	20	6 56	6	37 52	37 64	37 41	37 53	*37 53*	*37.58*	−0.0[illegible]
	E A	20	7 53	2	37 99	37 76	37 50	37 67	*37 73*	*37 67*	+0.0[illegible]
	F Z N	20	7 10	3	37 46	37 66	37 6[illegible]	37 31	*37 5[illegible]*	—	—

rectal temperature at each of the four depths and the mean rectal temperature (average of the four measurements) from 57 exp at 900 mkg/min performed on 4 subjects further shows that the scattering of the measurements at the different depths is extremely small In the recovery period the differences between the rectal temperatures measured at different depths were in some of the subjects found to be considerably larger than during the work period

Fig 2 Rectal temperature at four depths (12 cm △ 17 cm x, 22 cm ▫ 27 cm +) and deep oesophageal temperature ● Work intensity 450 mkg per min (Arm work) Subject T N Environmental temperature 20 C Average of results from 6 experiments



During arm work (see Fig 2 Table I and Table II) the rectal temperature measured at the four depths differed more than during leg work and the measurements at 27 and 22 cm depths tended to show lower values than at the lesser depths The number of experiments with arm work however was rather small (10—11 exp) and two of the subjects (E A and F Z N) showed also during leg work relatively large differences between measurements at different depths

The *oesophageal temperature* increased much faster during work than the rectal temperature Both in subject T N (Fig 1) and in the other subjects studied a new level was reached after 15 to 25 min of work This temperature level was from 0 23 C to 0 39 C lower than the rectal temperature at the end of the work period (see Table I) although the rectal temperature in some of the subjects was still increasing slightly In experiments with arm work also (Fig 2) the rise of the oesophageal temperature occurred much faster than that of the rectal temperature At the end of the work period however the two temperatures were about equal (Table I and Fig 2) In the recovery period the oesophageal temperature decreased much faster than the rectal temperature in both kinds of work the resting level being approached in 10 to 15 min

Table II Mean difference between rectal temperature measurements in four depths and mean rectal temperature (average of the four measurements)

	Depth in cm	number	Mean C	S D C	S E. ▫
Work with the legs 900 mkg/min	12	57	+ 0 026	0 07	± 0 009
	17	57	− 0 038	0 09	± 0 011
	22	56	+ 0 020	0 06	± 0 008
	27	57	− 0 013	0 06	± 0 008
Work with the arms 450 mkg/min.	12	11	+ 0 029	0 14	± 0 041
	17	10	+ 0 10[illegible]	0 15	± 0 047
	22	11	− 0 067	0 16	± 0 050
	27	10	− 0 073	0 16	± 0 051

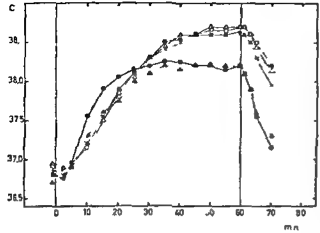


Fig 3 Rectal temperature ■ four depths (12 cm △ 17 cm x, 22 cm ▫ 27 cm +) deep oesophageal temperature ● and sublingual temperature ▲ Work intensity 900 mkg per min (Leg work) Subject T N. Environmental temperature 20 C Inspired air saturated with water vapor at body temperature Average of results from 4 experiments (Sublingual temperatures average of results from 3 experiments)

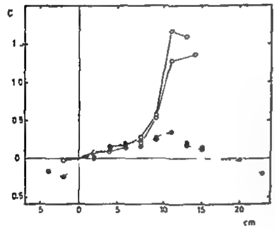


Fig 4 The temperature gradient through the oesophagus during work (900 mkg per min) Abscissa Distance from the routine place of measurement just above the diaphragm Ordinate Temperature difference Subject T N Environmental temperature 20 C. ○ Subject breathing room air (two experiments) ● Subject breathing air saturated with water vapor at body temperature (three experiments)

The difference between rectal and oesophageal temperatures found in leg work was not diminished in experiments in which the subject inspired air saturated with water vapor at body temperature (Fig 3 and Table I) In subject T N both rectal and oesophageal temperatures were increased about 0 3 C more than in the normal experiments while in the other subject (E A) water vapor breathing did not affect the temperature levels

Fig 4 shows measurements of the temperature differences between the standard measuring point just above the diaphragm and points at different distances from this in the oesophagus (a exp at a work intensity of 900 mkg/min) The measurements were performed from 30 to 60 min after the start of work In 3 of the experiments the subject breathed air saturated with water vapor at body temperature It is seen that in the lower part of oesophagus the temperature differences are very small and not influenced by breathing

water vapor. Five cm from the normal place of measurement the difference in temperature is only 0.15 C. Higher in the oesophagus the temperature difference during air breathing is much larger. At a distance of 12 cm above the normal level of measurement the temperature has decreased about 1.5 C. In the experiments with water vapor breathing, however, the decrease at this distance is only 0.35 C, and still higher in oesophagus the temperature is increasing.

Discussion

As mentioned in the introduction it has been maintained (MEAD and BONMARITO 1949) that the temperature in the deep part of the rectum is influenced by a cooling effect of venous blood returning from certain parts of the skin, and the authors recommended that rectal temperatures be measured at a depth of not more than 10 cm. In the present experiments with muscular exercise performed with the legs there were no systematic differences between rectal temperatures measured at depths of 12, 17, 22 and 27 cm and generally there was only a very small deviation in the measurements performed at the different depths (Table I and II). In some of the experiments with subjects E. A. and F. Z. N., though somewhat larger but unsystematic variations were found. In the following discussion, therefore, the mean rectal temperature calculated from the measurements at the four depths has been used for comparison with other internal temperatures.

The increase of the oesophageal temperature to the higher level during work and the decrease to the resting level in the recovery period occurred much faster than the corresponding changes in rectal temperature. This is probably due to a large heat capacity in proportion to blood flow of the tissues surrounding the rectum, while the temperature of the lower oesophagus better reflects the temperature of the blood in the heart and the aorta. This assumption agrees with the findings of COOPER and KENYON (1957) and CARLSTEN and GRIMBY (1957) that in resting man the deep oesophageal temperature is a good index of the aorta and the heart blood temperatures and of MELLETTE (1950) that the temperature changes in large blood vessels during work occur faster than the changes in rectal temperature.

The oesophageal temperature was at the end of work (50—60 min) considerably lower than the rectal temperature although the rectal temperature had not yet reached a plateau. It might be thought that this difference is due to a cooling of the oesophagus caused by the air flow through the respiratory passages. CRANSTON, GERBRANDY and SNELL (1954) found that in the resting condition the temperature in the oesophagus 30 cm from the lips was 0.5 lower than at a depth of 47 cm and further that a hyperventilation for 30 sec lowered the temperatures considerably. In the present experiments a temperature difference of 1.5 C was found between upper and lower part of the oesopha-

Table III Mean Δ TR ectal and mean Δ To s at 5 C 20°C and 30 C in ' steady state' of work of 900 mkg/min

Δ TRect l = Single TRectal — mean T°Rectal and Δ To = Single To — mean T°Oes where single TRectal is the average of the steady state measurements at 12 17 22 and 27 cm dephts in each experiment and single To is the steady state value of To s in each experiment Mean T°Rect l and mean T°O s are the mean of the "steady state values of TR l and To obtained at all environmental temperatures for each subject (cf Table I)

		numb r	Mean C	SD C	SE C
Δ TR l	5 C	18	+0 030	0 11	± 0 03
	20 C	21	−0 005	0 12	± 0 03
	30 C	18	−0 074	0 11	± 0 03
Δ To	5 C	18	+0 056	0 11	± 0 03
	20 C	21	−0 009	0 10	± 0 02
	30 C	18	−0 040	0 12	± 0 03

gus during work at 900 mkg/min (Fig 4) and, it seems possible that this large difference is caused by the continuous high ventilation (about 50 liters per minute) This possibility was examined in experiments in which the subject breathed air saturated with water vapor at body temperature It appeared that the major part of the temperature gradient was eliminated by breathing ater vapor The temperature in the deep part of the oesophagus however ained unchanged Furthermore in the 'steady state breathing water por did not diminish the difference (Table I) between the rectal and the esophageal temperature This temperature difference therefore can not be due to a cooling of the oesophagus In experiments in which the work was performed with the arms rather than the legs it was found (Fig 2 and Table I) that during the steady state of the work (50 to 60 min after the start of work) the oesophageal temperature and the rectal temperature were about equal It may therefore, be concluded that the difference between the two temperatures during work with the legs results largely from an elevation of the rectal temperature above the general internal temperature, and the fact that the rectal temperatures during leg work were the same at depths from 12 to 27 cm can best be explained by assuming that a large part of the contents of the pelvic cavity is equally heated by venous blood returning from the working muscles The temperature of these were shown by ASMUSSEN and BØJE (1945) to be considerably higher than the rectal temperature This conclusion further explains the earlier finding (ASMUSSEN and NIELSEN 1947) that at the same level of heat production the rectal temperature increases more during leg work than during arm work

In the experiments where water vapor at body temperature was inhaled it was possible to measure the sublingual temperature without the measurement

being influenced by a cooling of the oral cavity due to the respiration Under these conditions it was found, in the "steady state" of work, to be equal to the oesophageal temperature (see Fig 3) Since the deep oesophageal temperature in the steady state during arm work was found to be about equal to the rectal temperature and during water vapor breathing to the sublingual temperature the oesophageal temperature during work under these conditions seems to be a good index of the internal temperature of a large part of the body Whether the oesophageal temperature under normal conditions of respiration in which a considerable cooling of parts of the upper thorax and the neck takes place (cf the large temperature gradient Fig 4) is a satisfactory index of the temperature which influences the central thermoreceptors in the brain cannot be stated definetily Studies of this problem are in progress

The rectal temperature is, in agreement with earlier studies by NIELSEN (1938) practically independent of the environmental temperatures which varied between 5°C and 30 C Likewise the oesophageal temperature is practically independent of the temperatures of the environment On an average both the rectal and oesophageal temperatures were at 5 C a few hundredths of a degree higher and at 30 C a few hundredths of a degree lower than the mean temperatures (see Table III) The difference between the rectal temperature and the oesophageal temperature was also practically the same at different environmental temperatures suggesting that the temperature in the working muscles presumably is also essentially independent of the surrounding temperatures

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Acta physiol scand 1962 56 130—139

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The Effects of Adrenaline and Glucose on the Content of High-Energy Phosphate Esters in Substrate-Depleted Vascular Smooth Muscle

By

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Abstract

LUNDHOLM I and E MOHME LUNDHOLM *The effects of adrenaline and glucose on the content of high-energy phosphate esters in substrate depleted vascular smooth muscle* Acta physiol scand 1962 *56* 130—139 — The metabolism of high energy phosphate compounds was studied in substrate depleted bovine mesenteric arteries after addition of glucose alone and adrenaline plus glucose under anaerobic conditions Also determined was the lactic acid production in the suspension solution Glucose raised the content of ATP ADP and CrP as well as the lactic acid production From the lactic acid production estimated coincident increase in the synthesis of high energy phosphate compounds approximately onet hird was demonstrable in the muscle Adrenaline stimulated the lactic acid production further simultaneously with contraction of the muscle

Only a minor proportion (18 per cent) of the calculated amount of high energy phosphate compounds produced could be recovered however in the muscle the remainder was thought to have been consumed for the muscle contraction The creatine phosphate content of the muscle did not rise after addition of adrenaline On the other hand the ATP elevation tended to be greater and the ADP content lower than in the experiments with glucose alone The results are considered in relation to those of previous experiments which indicate that tone promoting drugs are capable of stimulating the contraction process and the carbohydrate metabolism via separate mechanisms

Investigations into the effect of tone increasing drugs on the contraction and carbohydrate metabolism of vascular smooth muscle indicated that despite a close correlation between the stimulating effects on the energy production and those on the contraction process each of those two factors could unde

certain experimental conditions be selectively inhibited (LUNDHOLM and MOHME LUNDHOLM 1962 a) Moreover, the energy consumption during muscular contraction was conspicuously high It was calculated that a total contraction of vascular muscle is only to a very small degree effectuated via the preformed high-energy phosphate compounds For a maximal contraction the muscle must draw upon a continuous energy production (LUNDHOLM and MOHME LUNDHOLM 1962 b)

The above conclusions were based largely upon quantitative estimates founded in turn on the lactic acid production of the musculature A potential source of error in these calculations was the possibility that the muscle did not utilize, for the contraction process the energy resulting from lactic acid production but instead augmented its high energy phosphate compound content and in this way stored a part of the energy produced — The following experiments which were conducted for the purpose of confirming or discounting this possibility disclosed no such storage of high energy phosphate compounds The results suggested moreover that the augmented energy production accompanying stimulation of the carbohydrate metabolism was reflected in an elevation of the adenosine triphosphate content whereas the consumption of high-energy phosphate compounds associated with the muscle contraction was reflected in a fall of the creatine phosphate content

The experiments were performed on bovine mesenteric arteries which were subjected to advanced substrate depletion under anaerobic conditions for the purpose of reducing their content of high energy phosphates to such a degree that resynthesis of the latter would be clearly demonstrable Glucose or adrenaline plus glucose was then added and the effect on the phosphate metabolism was studied

Methods

Although considerable advances have been made during the past decade with respect to determination of different organic phosphate esters in tissue extracts the method still involves a number of problems In the first place it is often difficult to separate completely the different phosphate compounds secondly these compounds are unstable particularly in acid milieu and thirdly they occur in such low concentrations that determination is difficult, especially in the case of smooth muscle Experiments on the latter are complicated moreover by the limited amounts of tissue available for analysis particularly in experiments where both the physiologic and pharmacologic reactions of the muscle preparations are to be studied Of the three methods available — chromatographic separation with ion exchangers and enzymatic or paper chromatographic procedures — we chose to use the last mentioned since it is not only suited for minute amounts of substance but permits extensive separation We employed the paper chromatographic method described by GERLACH DORING and FLECKENSTEIN (1958) The general mode of procedure was as follows (for details *vide* the last mentioned authors)

Bovine mesenteric arteries were obtained from abbatoir material Nine specimens approximately 10—12 mm long 15 mm wide and 0 8 mm thick were taken from a homogenous segment of artery and mounted in small plastic frames so that the

was uniformly distributed They were then immersed in 20 ml glucose free Tyrode solution saturated with N_2 at 38 C Changes in length of the preparations were recorded by an isotonic pen with a load of 10 g and with a ratio of 1 10 After 60 min 1 ml 10 per cent glucose was added to 3 of the preparations and glucose plus adrenaline at a concentration of 2 10^{-6} to 3 of them After a further 10 min all preparations were removed from the organ bath rapidly chilled in a mixture of ether and carbon dioxide snow and weighed Similarly treated preparations were thereafter combined in groups of 3 and ground with sand in a small iced mortar After extraction with 4—5 ml trichloroacetic acid the extract was centrifuged

Creatine phosphate (CrP) Of the measured amount of extract two 0 5 ml samples were taken for duplicate determination of creatine bound phosphate *ad modum* EGGLETON and EGGLETON (1929)

Total phosphate Two 0 1 ml samples were taken from the extract and burned with 30 N H_2SO at 180 C on a sand bath for at least 3 hours If at the end of that time a brown coloration persisted 30 per cent hydrogen peroxide was added in drops until the solution was clear The samples were then subjected to phosphorus assay Control solutions with a known P content were simultaneously tested

Organic phosphate (excluding creatine phosphate) On a 20 by 45 cm sheet of Whatman No 1 chromatographic paper 0 08—0 09 ml extract was applied with a microsyringe about 18 cm from one edge (A) and subjected to descending chromatography for 18—20 hours at 20 C with solvent I a consisting of 40 ml di isopropyl ether 30 ml n butanol and 20 ml 98 per cent formic acid Inorganic phosphate and creatine bound phosphate moved away from the starting line towards edge A while the organically bound fraction remained at the starting line The filter paper containing this fraction was cut out and after its area had been measured was incinerated with H^2SO as above and the P content determined The P content of a piece of clean filter paper equal size was concurrently determined and from the difference between the two the amount of organic phosphate was calculated Duplicate determinations carried out in each instance

Adenosine triphosphate diphosphate and monophosphate (ATP ADP AMP) On another sheet of Whatman No 1 chromatographic paper two doses of 0 08—0 09 extract were applied at points 18 cm distant from edge A following which chromatography was done as above After approximately 30 min drying the paper was cut away about 2 cm above the starting point counting from edge A This segment which contained inorganic and creatine phosphate was discarded The rest of the filter paper containing organic phosphate at the starting point was subjected to descending chromatography with solvent II a Chromatography this time was done in the direction of the opposite edge (B) Solvent II a had the following composition

18 mg 8-oxyquinoline dissolved in 8 ml ethanol 25 ml n propanol 27 ml n butanol 30 ml 25 per cent ammonia 10 ml distilled water and 0 2 ml 0 1 M EDTA in aqueous solution The chromatography proceeded for 33—36 hours at 26 C After drying for 30 min the filter paper was again subjected to descending chromatography this time with solvent II b for 24 hours at 26 C The composition of solvent II b was as follows 5 ml ethanol 25 ml n propanol 30 ml n butanol 30 ml 25 per cent ammonia and 10 ml distilled water The chromatographic paper was thereafter redried

When the chromatographic paper was illuminated by a mercury lamp with an emission of 265 mμ it was possible to localize the nucleotides The paper was placed against a thin screen brushed with a film of cadmium sulphide which fluoresced when illuminated with ultraviolet light Since the nucleotides absorbed this light they appeared as dark spots when the paper was viewed through the illuminated screen The spots thus delineated were cut out and the phosphate content determined as above

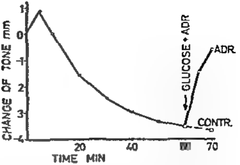

Fig 1 Effect of adrenaline on substrate depleted, glucose treated mesenteric arteries The preparations were suspended in glucose-free Tyrode solution under anaerobic conditions for 60 minutes, after which glucose plus adrenaline 2 10^{-6} was added.

The nucleotide spots which in no case exceeded three had the same R_f values as ATP ADP and AMP Additional two-dimensional chromatography of the observed nucleotide spots with solvent III was also attempted but no further division or reduction of the phosphate content could be obtained It is possible that the phosphorus compounds designated as ATP ADP and AMP included, to a certain extent, other high energy nucleotides If so the method is not sensitive enough to reveal those compounds in extracts from smooth muscle

The method permitted determination of a minimum of approximately 10 μg nucleotide P per gram muscle (= 0 1 μmoles ATP/g) Below this value not only were the nucleotide spots invisible under illumination with ultraviolet light but the P content of the chromatographic paper (about 0 07 μg per cm^2) interfered greatly with the P determination Assays of solutions containing known amounts of ATP ADP and AMP resulted in 95 per cent recovery

Inorganic phosphate was determined by subtracting creatine and organic phosphate from total phosphate

Unspecified organic phosphate was estimated from the difference between organic phosphate and ATP ADP and AMP

Phosphate determination was done *ad modum* MARTIN and DOTY (1948)

The lactic acid content of the suspension solution was determined *ad modum* FRIEDEMANN and GRAESER (1933) The available amount of trichloroacetic acid extract from the muscle did not suffice for estimation of the change in lactic acid content of the muscle thus only the lactic acid released into the Tyrode solution was determined

Free creatine was determined by the method of ENNOR and STOCKEN (1948)

Results

Tone The effect on vascular tone is evident from Fig 1 Following the initial spontaneous rise the tone gradually fell Addition of 0 5 per cent glucose after 60 min possibly inhibited this fall somewhat but caused no contraction After adrenaline 2 10^{-6} plus glucose the muscle contracted appreciably and at the end of 10 min had contracted by an average of 1 95 mm.

Lactic Acid Production Quantitative estimation of the lactic acid production could not be done in this investigation since only the lactic acid in the Tyrode solution was determined The amount of muscle extract was not sufficient for determination of variations in the lactic acid content of the muscle From the results of similar experiments carried out previously (LUNDHOLM and

Table I The effect of glucose and glucose + adrenaline on the phosphate metabolism and lactic acid

	Total P µmoles/g	Inorganic P µmoles/g	Creatine P µmoles/g
Normal muscle (n = 6)	12 7 ± 0 71	5 56 ± 0 95	0 56 ± 0 06
Substrate depleted muscl (n = 11)	16 46 ± 0 74	11 37 ± 0 76	0 69 ± 0 16
"Glucose treated muscle	16 10 ± 0 91	10 53 ± 1 29	1 00 ± 0 20
Difference glucose-depleted	—	−0 84 ± 1 06	0 31 ± 0 16
Glucose + adrenaline treated	15 63 ± 0 64	10 21 ± 0 82	0 73 ± 0 18
Difference: adrenaline-depleted	—	−1 16 ± 0 65	0 03 ± 0 11
Difference: adrenaline glucose	—	−0 33 ± 0 85	−0 29 ± 0 07 $p < 0\ 01$

P = phosphate p = probability that the effect was due to chance

MOHME LUNDHOLM 1962 b) it may be estimated that the total lactic acid production after adrenaline taking into account the variations in lactic acid content of the muscle would have been 30—50 per cent greater than that determined here The recorded values for lactic acid production after adrenaline glucose were therefore minimum figures Subject to this reservation Table I that the lactic acid production increased, after glucose by 4 3 es/g and after adrenaline by a further 1 2 µmoles/g The increase was tically verifiable

Phosphate Metabolites

Normal muscle Table I shows the averages ($n = 6$) from a run of experiments performed on mesenteric arteries which were kept for 60 min in oxygenated Tyrode solution containing 0 1 per cent glucose The values for the various phosphate metabolites are in close agreement with those from similar experiments on smooth muscle (LANGE 1955) In our table however, the P content is indicated in µmoles per gram To obtain the ATP or ADP content in µmoles/g the values must be divided by 3 or 2 as the case may be A striking observation was that even under aerobic conditions and with a substrate supply such a large amount of the nucleotides consisted of ADP and AMP In this respect a certain difference between smooth and striated muscle appears to exist (*cf e g* FLECKENSTEIN *et al* 1954) Determination of free creatine *ad modum* ENNOR and STOCKEN (1948) showed a mean content of 4 7 ± 0 23 µmoles/g ($n = 6$) Not more than approximately 10 per cent of the creatine in vascular muscle was phosphate bound Since the amount of inorganic phosphate in vascular muscle was also strikingly high (5 6 µmoles/g) it might

production of mesenteric arteries

Organic P –CrP μmoles/g	ATP P μmoles/g	ADP P μmoles/g	AMP P μmoles/g	Unspecified organic P μmoles/g	Lactic acid production μmoles/g
7.78 ± 1.06	2.16 ± 0.44	1.84 ± 0.50	1.24 ± 0.24	3.3 ± 1.5	—
4.43 ± 0.58	0.20 ± 0.12	1.01 ± 0.50	1.91 ± 0.48	1.27 ± 0.63	6.76 ± 0.54
4.80 ± 1.01	1.04 ± 0.77	2.10 ± 0.75	0.87 ± 0.15	0.78 ± 0.51	11.12 ± 1.63
0.36 ± 0.89	0.83 ± 0.26	1.06 ± 0.34	−1.05 ± 0.43	—	4.36 ± 1.3
	$p < 0.01$	$p < 0.01$	$p < 0.05$		$p < 0.01$
4.79 ± 0.81	1.65 ± 0.25	1.04 ± 0.16	1.01 ± 0.38	0.97 ± 0.83	12.29 ± 1.87
0.35 ± 0.90	1.45 ± 0.31	0.02 ± 0.39	−0.91 ± 0.21	—	5.53 ± 1.53
	$p < 0.01$		$p < 0.01$		$p < 0.01$
−0.01 ± 0.81	0.62 ± 0.35	−1.05 ± 0.60	−0.14 ± 0.34	—	1.18 ± 0.46
					$p < 0.05$

well be asked whether under more favourable and "basal" conditions a greater proportion of creatine and phosphate would not have been esterized. WOLLENBERGER, KRAUSE and WAHLER (1958) in experiments on heart found that the more refined and meticulous the technique the greater the proportion of creatine esterized; they reported a maximum value of 50 per cent. The question is of interest and requires further investigation.

Substrate depleted muscle. With substrate depletion and anaerobic conditions the ATP and ADP content decreased, but the AMP content increased in relation to normal muscle. The values indicated in table I are the means from 11 experiments. Even organic phosphate decreased, whereas inorganic phosphate increased. Since, however, the two groups of preparations were from different mesenteric arteries no direct comparison is possible. The changes shown by phosphate metabolites under anaerobic conditions and substrate depletion are well known from experiments on other types of tissues.

Glucose treated muscle. Following the addition of glucose, resynthesis of ATP and ADP occurred, whereas the AMP content fell. The CrP content increased. The content of organic phosphate tended to rise, while that of inorganic phosphate tended to fall. In these two instances, however, the changes were not significant. Here too, the pattern was typical in that addition of glucose led to partial resynthesis of the high-energy phosphate compounds.

It may be asked to what extent the increased lactic acid production after addition of glucose was utilized by the muscle for resynthesis of its high-energy phosphate compounds. With the formation of 1 mole lactic acid from glucose 1 eq ~ P (high-energy phosphate bond) is synthesized. In biologic terms ATP contains 2 eq ~ P per mole, while ADP and CrP each contain 1 eq ~ P

It can be computed from these values that after addition of glucose the ~ ph content increased by 1 42 μeq/g concurrently with a rise of 4 36 μmoles/g in the determinable production of lactic acid Approximately one third of the lactic acid elevation after glucose was thus utilized for resynthesis of high energy phosphate compounds

Muscle treated with adrenaline plus glucose Resynthesis of high-energy phosphate compounds was observed after adrenaline plus glucose as well as after glucose alone, though certain definite differences were demonstrable Of most significance was the fact that the creatine bound phosphorus content did not increase in the adrenaline experiments, the difference between adrenaline and the glucose experiments being, in this respect statistically verifiable The ATP content, on the other hand, tended to show a greater increase in the adrenaline than in the glucose experiments while the converse was true for the ADP content The effects considered separately were not statistically verifiable *When however the quotient* $\frac{\text{ADP}}{\text{ATP}}$ *was calculated it was found to have in*creased in 9 of 11 experiments The mean value in the glucose test was 0 79 $\pm$ 0 23 and in the tests with glucose + adrenaline *1 64* $\pm$ *0 32* *The* increase 0 86 $\pm$ 0 27 (P < 0 01) was statistical significant, which *indicated* that a synthesis of ATP from ADP had occured in the adrenaline experiments

The total synthesis of ~ ph in the latter experiments was 1 0 μmole/g while the increase in lactic acid production amounted to 5 53 μmoles/gram. Although the lactic acid production was 1 18 μmole/g greater in the adrenaline [illegible]n in the glucose experiments the recorded synthesis of high energy phosphate [illegible]onds was 0 42 μeq/g lower Nor was the content of other unidentified organic phosphate compounds demonstrably higher in the adrenaline experiments when compared with the substrate depleted preparations

Discussion

The primary aim of this investigation was to ascertain whether the stimulation of the carbohydrate metabolism induced by tone increasing drugs was associated with an elevated content of high-energy phosphate compounds or whether these compounds were utilized by the muscle during its contraction process The latter seemed to be the case for even though the lactic acid production was at least 1 [illegible] μmoles/g greater in the adrenaline than in the glucose experiments the synthesis of high energy phosphate bonds was 0 4 μeq lower A hypothetical possibility was that adrenaline induced synthesis of a readily hydrolyzable phosphate compound which was subsequently determined as inorganic phosphate No signs of such behavior were manifest however

It is more difficult to account for the observed relative shifts in the various phosphate fractions after adrenaline According to the classic conception the muscle utilizes ATP primarily for contraction after which *ATP is re*[illegible]nthesized from ADP with the aid of CrP in conformity with *Lohmann's*

(1935) reaction $CrP + ADP \rightleftharpoons ATP + Creatine$ NODA, KUBY and LARDY (1954) using ATP creatine transphosphorylase from skeletal muscle found that the equilibrium in this reaction was influenced by the pH and the Mg^{++} concentration A pH fall shifted the equilibrium to the right *i e* towards an increase of the ATP content as did a rise in the Mg^{++} concentration — Since in our experiments adrenaline stimulated the lactic acid production of mesenteric arteries and also raised the lactic acid content of the muscle it was probable that a certain pH fall occurred after adrenaline It may be asked how great a pH fall was required for a change in the ATP CrP content of such magnitude as that observed in the adrenaline experiments Calculation of the apparent equilibrium constant $K = \frac{CrP \; ADP}{ATP \; Cr}$ from the values in Table I on the assumption that $Cr = 4\ 7$ µmoles/g gave a value of $K = 0\ 43$ for the glucose experiments and of 0 09 for the adrenaline glucose experiments From the data reported by NODA *et al* (1954) it may be computed that such a change of K will coincide with a pH alteration of 0 6—0 9 units If therefore ATP creatine transphosphorylase from smooth muscle has the same pH optima as enzyme from striated muscle — as is suggested by GEIGER s (1956) studies on fowl gizzard — a pH alteration of the abovementioned magnitude could account for the change in the ATP CrP concentrations Since however such pH changes after adrenaline have neither been demonstrated nor are *a priori* probable this explanation of the observed changes in the phosphate fractions is hardly plausible

The possibility that an increased amount of nucleotid charges was formed in order to neutralize positive charged adrenaline taken up by the muscle (review HAGEN and BARRNETT 1960) was considered The total charge in experiments with glucose was 12 20 µeq/g and in experiments with adrenaline + glucose 11 74 µeq/g This explanation of the observed effect seemed thus not probable

The view that ATP is primarily utilized in muscle contraction and is secondarily resynthesized with the aid of CrP has been criticized on the grounds that the contraction process is not demonstrably accompanied by changes in the ATP content It has been possible to show however an increase of the inorganic phosphate content in connection with muscle contraction at which an as yet unidentified phosphorus ester is broken down Creatine phosphate is assumed to be capable of rephosphorylizing this hypothetical ester (for review *vide* DAVIES CAIN and DELLUVA 1959) It has been shown that creatine phosphate may be synthesized by muscle extracts even in the absence of the adenylic acid system (CORI *et al* 1956) — an observation which suggests that creatine phosphate may participate in the phosphorylation processes more directly than via ATP—ADP

BORN (1956) found that the ability of smooth muscle to exert tension was dependent on the CrP content but not on the ATP content A similar finding was recorded by CSAPO (1960) LANGE (1955) reported that adrenaline and

acetylcholine while contracting smooth muscle from rabbit stomach raised the ATP content. From the cited investigations as well as our own, it might be surmised that the contraction process in smooth muscle was associated with utilization of CrP. Concurrently, however, synthesis of ATP took place. One is tempted to hypothesize a connection between this latter effect and the stimulation of the carbohydrate metabolism which is produced by tone promoting drugs even when the contracting effect is blocked. The metabolism of high-energy phosphate compounds in vascular muscle would accordingly reflect, via elevation of the ATP content, the stimulation of the energy production to which such drugs give rise and, via depression of the CrP content, the increase of the energy consumption which is associated with muscle contraction. Further investigations into the metabolism of high-energy phosphate compounds in smooth muscle on selective stimulation of the contraction process or of the carbohydrate metabolism might well shed additional light on this problem.

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The Effect of Electrical Stimulation in Nucleus Ruber on the Response to Stretch in Primary and Secondary Muscle Spindle Afferents

By

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Abstract

Appelberg B *The effect of electrical stimulation in nucleus ruber on the response to stretch in primary and secondary muscle spindle afferents* Acta physiol scand 1962 *56* 140—151 — In cats anesthetized with Nembutal the activity in muscle spindle afferents was recorded in thin dorsal root filaments The conduction velocity of each fibre recorded from was determined The value of 72 m/sec was used for separating primary and secondary afferents The effect of electrical stimulation in the red nucleus on the adapted discharge to a steady stretch of the muscle under study was tested It appeared that while the activity in primary afferents was as a rule strongly inhibited the secondary discharge was only slightly or not at all influenced by the rubral stimulus The dynamic burst of activity in primary afferents seen during the application of a slow extension of the muscle was also studied The rubral stimulation as a rule caused an increase in the dynamic response although the static discharge was inhibited before as well as after the application of stretch The results are discussed in relation to anatomical and physiological knowledge about muscle spindles The conclusion is reached that the red nucleus has an inhibitory influence upon the gamma supply to nuclear chain intrafusal fibres but does not affect the motor innervation of the nuclear bag fibres

In a previous investigation (Appelberg 1962) it was shown that the red nucleus depressed the afferent discharge from muscle spindles in muscles of the contralateral hindleg This depression was shown to be due to *inhibition* of efferent gamma discharge

It was noted during the course of these experiments that occasional spindles could not be influenced by the central stimulus

It is known from histological investigations (BOYD 1958 1959 1960 1961 BARKER and IP 1960) that the muscle spindles consist of intrafusal muscle fibres of principally two different types nuclear bag (NB) and nuclear chain (NC) fibres These authors have also shown that the two types of intrafusal fibres have separate motor innervation and that the sensory innervation of the fibres is fundamentally different The NB fibres are thus supplied almost exclusively with so called primary sensory endings although some secondary endings may be found in the myotube region of these fibres The NC fibres on the other hand have a primary innervation as well as the majority of the secondary sensory endings

It was considered possible that the red nucleus might exert inhibition upon one type of gamma efferents only either those destined for NB fibres or those running to NC fibres For this reason it was decided to investigate the rubral effect upon primary and secondary muscle spindle afferents

Methods

15 cats anesthetized with Nembutal were used for the experiments Muscle spindle activity in the right gastrocnemius or the semitendinosus muscles was recorded in thin dorsal root filaments The experimental arrangement was essentially the same as that described by APPELBERG (1962)

Every dorsal root filament containing a functional single fibre was tested in the following manner

1 The response to a muscle twitch caused by an electric shock to the muscle nerve was recorded The pause during contraction (MATTHEWS 1933) served as a criterion for muscle spindle afferents

2 Conduction velocity was determined by measuring the latency of the spike over the distance muscle nerve — dorsal root which was measured after each experiment by dissection. When calculating the conduction velocity no time for the initiation of the impulse at the stimulating site was allowed for

3 The behaviour of the afferent discharge during a rapid and then maintained stretch of the muscle was also studied According to the type of response the afferent fibre was classified as having dynamic and static sensitivity or "static sensitivity only By dynamic sensitivity is meant the ability of a receptor to react phasically to the change in stimulating intensity and thus respond with a burst of activity at onset of stretch For most filaments this classification was made merely by listening to the loudspeaker while pulling out the muscle by hand or with the aid of an apparatus which will be described below In some cases the result was recorded on running film (100 mm/sec)

4 The effect of electrical stimulation in the red nucleus was tested Usually a load was then applied to the muscle so as to set the receptor recorded from at a fairly high level of activity The spindle was always allowed to adapt to the load for several minutes before the rubral effect was tested Thus the effect of the central stimulation was observed with a static discharge as indicator

When studying the effect of electrical stimulation in the red nucleus the discharge of the fibre was recorded in successive sweeps at intervals of 0 7 sec which usually had a duration of 160 msec As a rule twenty sweeps were taken for each fibre Of these the ten first were controls while the rest served to investigate the effect of rubral

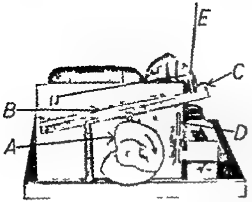

Fig 1 Photograph of the pulling device used for extending the muscle A is a cam rotating in the direction of the arrow The upward and downward movements of the lever B governed by the cam curve is transferred to the string E which stretches the muscle The rate of stretch is adjusted by changing the length of the lever at C The screw D can be used to stop the lever before reaching the bottom of the cam curve and thereby also the length of pull may be varied

stimulation By counting the number of spikes in the sweeps before and during stimulation the effect could be expressed in per cent

In the beginning of every experiment the strength of the stimulus to the red nucleus was adjusted so as to yield maximal inhibition of the discharge in a primary afferent with minimal motor effects seen in the preparation Small movements of whiskers, ears and eyes were sometimes caused by the stimulus The sensitive myograph used for checking on muscle tension never revealed any variations in tension accompanying the central stimulus

In six of the cats the effect of rubral stimulation upon the dynamic response of primary spindle afferents was studied For this it was of great importance that rate and amount of extension should be properly standardized Therefore a special apparatus was used designed by Mr L. Strom and built by Mr I Andersson Kungl Veterinarhögskolan This pulling device the main details of which are shown in Fig 1 consisted of an electric motor geared down to about one revolution in ten seconds. The gearbox drove a cam (A) which influenced a lever (B) to which the string to the muscle was attached By varying the length of the lever (at C in Fig 1) the rate of pull could be changed The length of pull was set by a screw (D) stopping the lever before it reached the bottom of the cam curve The design of the cam curve was such that a linear pull was achieved The string from the pulling device was always connected to the muscle via the myograph spring and each change in stretch of the muscle was therefore recorded on the screen

By changing the rate and degree of stretch the optimum intensity of stretch for each spindle investigated could be achieved Thereby a clearcut dynamic response to stretch was regularly obtained

The dynamic response to stretch in primary muscle spindle afferents was recorded on running film (100 mm sec) Usually four control records were photographed for each afferent The effect of rubral stimulation was then studied in another four records.

The frequency of the discharge in the fibre was determined on the film by counting the number of impulses in successive periods of 0 1 sec This was made before stretch began during the application of stretch and also for a short time after completion of stretch When making the diagrams presented below the mean frequency values in corresponding parts of the four records for each fibre were used

The myograph used was the one previously described (Appelberg 1962) At maximum sensitivity a 1 gram load on the myograph spring gave a deflection of 4 5 mm of the myograph beam

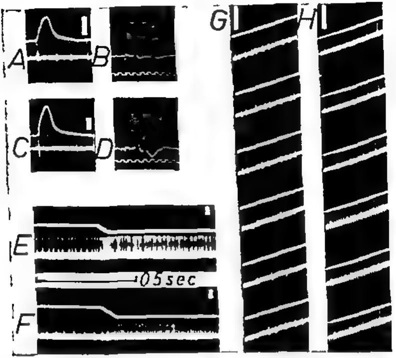

Fig 2 Response to different stimuli in one primary (A, B E, G) and one secondary (C, D F H) muscle spindle afferent A and C show the pause in the discharge during muscular contraction caused by electrical stimulation of the muscle nerve Time 20 msec Calibration for myograph beam 200 g
B and D give the latencies of the primary (first spike in B) and the secondary (second smaller spike in D) afferent spikes recorded in dorsal root on electrical stimulation of the muscle nerve. Conduction distance 107 mm Time 1 msec E and F demonstrate the behaviour of the discharge in the two afferents during the application of stretch Myograph calibration 400 g G and H show the inhibitory effect of electrical stimulation in the red nucleus on the discharge in the two afferent fibres In both cases the three first sweeps are controls while the three following were obtained during rubral stimulation (note stimulating artifacts) Time each sweep comprises 160 msec Myograph calibration [illegible] g

Results

1 The rubral effect on the static discharge of primary and secondary afferents

A total of 65 muscle spindle afferents were analyzed according to the above mentioned principles If the dividing line for conduction velocity between primary and secondary spindle afferents is set at 72 m/sec (Hunt 1954) 15 of the fibres studied were secondary and 50 primary

In Fig 2 is shown the reactions to the tests applied for one primary (A B E G) and one secondary (C D F H) afferent Records A and C demonstrate the typical spindle discharge in both cases with the pause during a contraction of the muscle B and D are the records for conduction velocity det

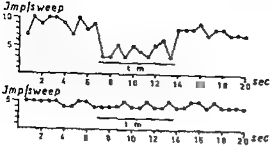

Fig 3 The inhibitory effect of electrical stimulation in the red nucleus on the discharge of a primary (A) and a secondary (B) ending The curves were obtained by counting the number of spikes in 10 sweeps before 10 sweeps during and 10 sweeps after the period of stimulation

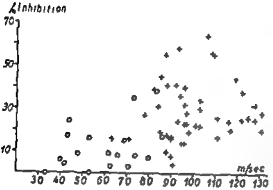

Fig 4 The inhibitory effect of the rubral stimulation in 65 muscle spindle afferent fibres plotted against conduction velocity of each fibre Plus symbols indicate fibres responding with a dynamic burst during application of stretch of the muscle Circle symbols indicate lack of such response

In both cases the conduction distance was 107 mm The latency of 24 msec for the primary and 2 46 msec for the secondary afferent yields conduction velocities of 87 and 43 m/sec respectively

In Fig 2 E and F is illustrated the behaviour of the discharge in the two fibres during a rapid and then maintained stretch of the muscle The primary afferent discharge (E) shows a burst of activity during the dynamic phase Upon completion of stretch it rapidly adapts to a new level of activity slightly above the initial one The secondary discharge (F) yields no burst during the dynamic phase of stretch but rapidly changes its level of activity to a new higher frequency which only slowly adapts as stretch is maintained This difference between primary and secondary afferents first noted by COOPER (1959 1961) was found to be a constant feature of the whole material of fibres COOPER's observation confirmed by LUNDBERG and WINSBURY (1960) HARVEY and MATTHEWS (1961) for deefferented spindles and by JANSEN and MATTHEWS (1961) for spindles with intact efferent innervation thus also by the present results seems to provide a firm basis for differentiation of primary and secondary spindle afferents

Fig 2 G and H finally illustrate the response of the primary and the secondary afferent to electrical stimulation of the red nucleus Only six out of the twenty sweeps recorded for each filament are shown A clearcut difference in degree of inhibition of the activity in the two fibres is evident In fact the primary

discharge was inhibited by 55 % while the frequency of the discharge in the secondary fibre only decreased by 17 %. The inhibitory effect of rubral stimulation on the discharge in the two fibres shown in Fig. 2 is illustrated graphically in Fig. 3. Also the activity in ten sweeps after the stimulation is shown.

The findings demonstrated in Figs. 2 and 3 are typical for the majority of the investigated spindles. In Fig. 4 is presented a diagram where the percentage of inhibition for each fibre investigated is plotted against conduction velocity. Plus symbols indicate fibres responding with a dynamic burst to stretch. It is evident that the majority of primary afferents revealed dynamic sensitivity while the secondary did not.

The most interesting feature in the diagram of Fig. 4 is, however, the marked quantitative difference in the inhibitory influence exerted by the red nucleus on the discharge in primary and secondary spindle afferents. The inhibition of primary afferents ranged between 4 and 64 % with a mean of 29 % while the corresponding figures for secondary fibres were 0 and 24 % with a mean of 9 %. The inhibition of the secondary discharge is in fact often so small that it may be due only to random frequency variations. The fact that the circles in the diagram without exception fall above the zero line and that some of them show definite inhibition is clearly in favour of a genuine though small effect also on the secondary discharge.

2. The rubral effect on the dynamic discharge of primary afferents

During the course of the experiments JANSEN and MATTHEWS (1961) presented results according to which the dynamic and static components of the response to stretch of primary spindle afferents were differentially controlled by their gamma fibres. The authors suggested that the NB primary endings would provide the dynamic and some static sensitivity, while most of the static sensitivity would be served by NC primary endings. Experiments by GRANIT and VAN DER MEULEN (1962) lend themselves to a similar interpretation.

This hypothesis made it interesting to study not only the effect of rubral stimulation upon the static discharge of primary and secondary afferents but also to pay special attention to possible effects on the dynamic burst as seen in primary afferents at onset of extension.

The effect of rubral stimulation upon the dynamic response to stretch was studied in 10 primary spindle afferents. It appeared that although the adapted static response to a given amount of extension of the muscle was always inhibited by the red nucleus, the dynamic component was affected in quite a different way. In most of the afferents studied the dynamic burst significantly increased during rubral stimulation. In some fibres the burst was practically uninfluenced, but a decrease in dynamic sensitivity was never observed.

In Fig. 5 is shown results obtained in two different spindle afferents. The afferent studied in A and B had a conduction velocity of 90 m/sec and its static response to stretch was inhibited 16 % by electrical stimulation in the

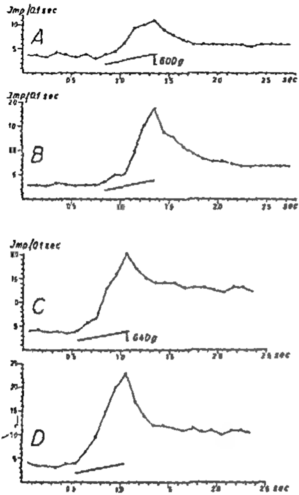

Fig 5 The effect of electrical stimulation in the red nucleus on the dynamic response in two primary muscle spindle afferents A and C represent the control records for the two fibres B and D show the effect of the rubral stimulation Each point in the curves was obtained by calculating the mean of the number of impulses in corresponding 0 1 sec parts in 4 different film records The stretch applied extend d the muscle 12 5 mm The timecourse of the extension is indicated by the oblique line in each diagram

red nucleus In the control records the mean values of which are diagrammatically represented in A the response to the slow stretch applied was seen to have a rather small dynamic component During simultaneous rubral stimulation the dynamic response increased (B) The increase in this case was 22 % (calculated by comparing the top of the dynamic burst with the mean discharge before stretch) It was further noted that as the new stretch was maintained the dynamic response rapidly adapts but at the end of the curve presented there is still enough of it left to mask the inhibition of the static response Inhibition was not revealed until stretch had been maintained for 15—20 sec

The spindle afferent represented by the two curves in C and D of Fig 5 behaved in a somewhat different way The conduction velocity of this fibre was 75 m/sec and the inhibition of its discharge before stretch was 13 %

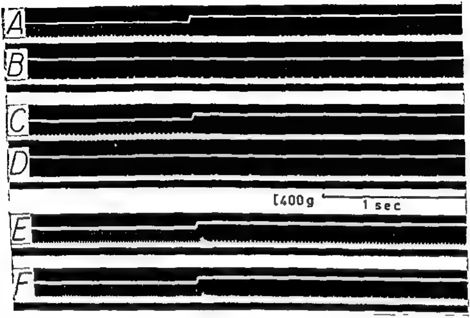

Fig 6 The behaviour upon release of stretch in one primary (A B C D) and one secondary (E F) afferent
A and B which are continuous records serve as controls for the primary afferent During C and D the red nucleus was stimulated
Note between C and D about 3 sec were omitted when making the figure During this period no discharge was seen E is the control record for the secondary fibre During F the red nucleus was stimulated

During rubral stimulation the dynamic burst was increased 5 % The dynamic response of this fibre was however of a very phasic character and was rapidly cut short by the rubral stimulation (D) The inhibition of the static discharge was in this case already seen 0 1 sec after completed extension The static discharge was then inhibited to approximately the same degree as during the period preceding the stretch These findings indicate that the dynamic response may sometimes have a static component extending the timecourse of the burst over several seconds

An interesting observation which was regularly made in connection with these experiments is demonstrated in Fig 6 which shows the behaviour upon release of stretch in one primary (A and B) and one secondary (E) afferent As was first observed by Adrian and Zotterman (1926) in the frog and later demonstrated in mammals by Matthews (1933) the activity of primary spindle afferents is for a short while depressed when the muscle is suddenly released from a stretch (A and B) The activity then gradually returns to a level corresponding to the new length of the muscle In secondary afferents on the other hand this depression is generally very small or lackin

was first described by COOPER (1959, 1961) for spindles with an intact gamma innervation and confirmed by HARVEY and MATTHEWS (1961) for deefferented spindles

When the red nucleus was stimulated simultaneously with release of extension it appeared that the reduction in frequency of discharge upon release was much more marked for the primary afferent (C and D) The discharge is seen to disappear wholly for several seconds Still more interesting is the fact, that also in the secondary afferent (F) a well marked depression upon release appeared upon consequent stimulation in the red nucleus

Effects similar to those described in Fig 6 were noted in all ten primary afferents investigated and also in 3 secondary afferents studied in this way

Discussion

The main result of the present investigation is the observation that the discharge in secondary muscle spindle afferents is *far less susceptible to inhibition* by stimulation in the red nucleus than the primary afferent discharge In addition it was found that the dynamic and static components in the response of primary afferents are differently influenced by the rubral stimulation

It has been shown previously (APPELBERG 1962) that electrical stimulation in the red nucleus decreases the discharge in gamma efferent fibres The fibres are motor for the intrafusal muscle fibres in the spindles Therefore it must be assumed that the intrafusal fibres being deprived of their motor innervation during rubral stimulation will relax *i e* the tension in these fibres will decrease Thereby also the afferent discharge from the sensory endings on these fibres will decrease

The observation that electrical stimulation of the red nucleus causes a decrease of the primary static response and a simultaneous increase *of the* dynamic indicates that these two components have different origin within the spindle The findings therefore supplement the results of JANSEN and MATTHEWS (1961) and GRANIT and VAN DER MEULEN (1962) *The red nucleus* evidently inhibits the gamma activity to one type of intrafusal fibres only namely the ones from which the static component of the primary *response* originates From anatomical investigations it is known that primary and secondary endings occur together on NC fibres while primary endings dominate on NB fibres (c f the schematic drawing of a spindle in Fig 7) As only the primary afferent discharge is known to reveal a dynamic component to stretch it is reasonable to believe that this component emanates from primary endings on the NB fibres Both primary and secondary endings on NC fibres *on the* other hand respond tonically to stretch It may thus be concluded *that* electrical stimulation in the red nucleus inhibits the gamma discharge to NC

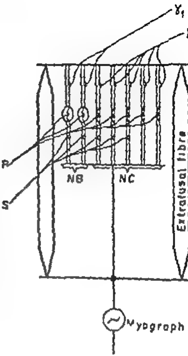

Fig 7 Schematic drawing of a muscle spindle coupled in parallel to two extrafusal muscle fibres The spindle consists of 2 NB fibres (to the left) and 5 NC fibres (to the right) P is the primary afferent fibre from the spindle S the secondary afferent Two of the NC fibres are shown lacking secondary endings This is in agreement with anatomical knowledge

fibres thereby decreasing the discharge of primary as well as secondary endings here

As a result of the parallel coupling between the two types of intrafusal fibres (Fig 7) a relaxation of the NC fibres will increase the load on the NB fibres This seems to explain the increase in dynamic sensitivity caused by the rubral stimulation

The load taken up by the NB fibres as the NC fibres relax will not only cause an increase of the dynamic sensitivity of the NB primary endings It will also cause a slight increase in the discharge of secondary endings in the myotube region of the NB fibres If it may be assumed that these endings respond tonically in contrast to NB primary endings this may serve as an explanation of the fact that the over all secondary discharge is much less influenced by the rubral stimulus The decreased activity of NC secondary endings will be counteracted by a simultaneous increase of NB secondary endings

The different behaviour of the discharge in primary and secondary afferents upon release of stretch is difficult to explain The strong depression of the primary discharge (Fig 6 A B) is likely to depend upon an inability of the intrafusal fibres underlying tonically active primary endings to follow a rapid change in extrafusal length During rubral stimulation the NC fibres will

become still less efficient in changing their length and therefore the depression upon release will be exaggerated (Fig 6 C, D) and marked also in the secondary response (Fig 6 F)

The fact that the discharge in secondary fibres shows a considerably less slowing when stretch is released (Fig 6 E) suggests that some of the secondary endings are situated on intrafusal fibres which follow extrafusal length changes more rapidly Provided that the myotube region of NB fibres may be assumed to have such properties secondary endings here will tend to counteract the depression in the discharge of NC secondary endings

In the present interpretation of the results the secondary endings on the myotube region of the NB fibres are assumed to play a very important role and this part of the NB fibres is supposed to have characteristics quite different from the bag region and also different from those of the NC fibres An alternative hypothesis would be the assumption of a third type of intrafusal fibre with secondary endings only, elastic properties different from the NC fibres and a gamma supply which is not influenced by the red nucleus

The results obtained in the present investigation thus strongly indicate that the red nucleus exerts an inhibitory action upon the gamma supply to the NC intrafusal fibres The consequence of this is a lowering of the static sensitivity of primary endings Due to the mechanical coupling between the intrafusal fibres the static sensitivity of secondary endings is influenced to a much less degree and the primary dynamic sensitivity is increased or uninfluenced

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Acta physiol. scand. 1962. 56 152—156

From the Institute of Occupational Health, Helsinki Finland

Distribution of Intravenously given Cholesterol-4-C^{14} between Rat Serum Lipoprotein Fractions

By

ESKO KARVINEN and MATTI MIETTINEN

Received 27 February 1962

Abstract

KARVINEN E. and M. MIETTINEN *Distribution of intravenously given cholesterol-4-C^{14} between rat serum lipoprotein fractions* Acta physiol scand 1962 56 152—156 — Distribution of cholesterol-4-C^{14} in alpha$_1$, alpha$_2$ and beta lipoprotein fractions separated by paper electrophoresis was studied from 2 minutes to 56 hours after intravenous administration of cholesterol-4 C^{14} in Tween 20 saline emulsion. The percentage of cholesterol-4-C^{14} in the lipoprotein fractions was about the same as the percentage of chemically determined cholesterol in these fractions The distribution was not changed with time. During the first half hour after the injection of cholesterol 4-C^{14} the activity in all fractions decreased rapidly but after the initial decline the apparent turnover time of cholesterol-4-C^{14} in the different lipoproteins was about 52 hours These findings support the view that the cholesterol-4-C^{14} containing compounds formed were true lipoproteins

Distribution of orally given cholesterol-4 C^{14} in paper electrophoretically separated alpha$_1$ alpha$_2$ and beta lipoproteins of rat serum is about the same (KARVINEN and MIETTINEN to be published) as the distribution of chemically determined cholesterol in these lipoprotein fractions (MIETTINEN 1957) The apparent turnover time of cholesterol-4-C^{14} given orally to the rat was estimated to be about 79 hours in alpha$_1$ alpha$_2$ beta and chylomicron fractions (KARVINEN and MIETTINEN unpublished)

It was considered worthwhile to study the distribution of intravenously given cholesterol-4-C^{14} between the serum lipoproteins and to follow the disappearance of the labeled compounds thus formed

Table I Estimated relative specific activities[1] of cholesterol bound in the different lipoprotein fractions

Time after cholesterol-4-C^{14} given iv	α	α	β	"Chylomicron
2 minutes	25 7	29 2	35 0	141 6
30 minutes	4 6	5 4	6 0	21 2
8 hours	3 0	5 0	2 8	10 6
24 hours	1 8	1 9	2 1	8 9
32 hours	1 8	1 9	2 3	8 5
48 hours	1 8	1 8	1 9	12.2
56 hours	1 3	0 9	1 7	5 9

[1] Total radioactivity of the lipoprotein bound unsaponifiable lipid divided by the amount of cholesterol in each fraction determined colorimetrically (Miettinen 1957)

Material and Methods

Male rats of Wistar strain weighing 200 to 300 g were caged individually and given a stock diet for 3 weeks ad libidum Then the rats were fasted overnight. Around 8 o clock on the following morning 2 μc of cholesterol-4 C^{14} in a Tween 20 saline emulsion were injected intravenously under ether anesthesia in the tail vein The cholesterol-4-C^{14} emulsion was prepared according to MEIER SIPERSTEIN and CHAIKOFF (1952) A benzene solution of cholesterol-4 C^{14} (Radiochemical Centre Amersham England) containing 8 μc of activity in 126 μg of cholesterol was taken to dryness A drop of Tween 20 (polyoxyethylene sorbitan monolaurate) was first added and the cholesterol was dissolved in ethanol The ethanol was evaporated on a steam bath and the viscous solution of cholesterol in Tween 20 was then diluted to 2 ml of volume with saline Two minutes after the injection the first blood sample of approximately 1 ml was taken under ether anesthesia from the tail proximal from the site of injection Blood samples of 4 rats were then taken from the tail vein 0 5 8 24 32 48 and 56 hours after the injection of the cholesterol-4 C^{14} emulsion Blood samples of another 5 rats were taken 0 5 9 25 33 49 73 97 145 169 193 and 217 hours after the injection of 0 8 μc of the cholesterol-4-C^{14} emulsion The fractionation of the serum lipoproteins was carried out according to the paper electrophoretic method of NIKKILA (1954) as modified and described elsewhere (MIETTINEN 1957)

The electrophoresis paper was cut into stripes containing albumin plus α_1 globulin + α_1 lipoprotein α_2 globulin plus α_2 lipoprotein β globulin + β lipoprotein and the area around the starting line containing chylomicrons The stripes were boiled in 10 % ethanolic KOH for 2 hours The unsaponifiable lipid was taken up in petroleum ether and washed with 50 % ethanol and water The unsaponifiable lipid was transferred to steel planchets and assayed for radioactivity using a thin mica window Geiger tube (20th Century EW 3 H) and an EKCO Automatic Scaler N 530 F

Results

In Fig 1 the total radioactivity found in the unsaponifiable lipid fraction of α_1 α_2 and β lipoprotein and the "chylomicron" fraction (= the residual radioactivity in the serum lipid fraction absorbed around the starting line of

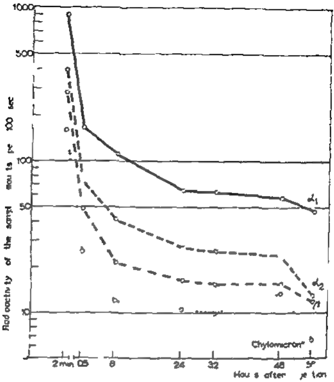

Fig 1 Activity time relationship of α β and β lipoprotein and chylomicron fractions of rat serum after intravenous injection of cholesterol-4 C^{14}

the electrophoresis paper) is shown as a function of time Relative specific activities in the lipoprotein fractions are presented in Table I It is noted that the residual radioactivity not bound to the lipoproteins is very small already 2 min after the intravenous administration of cholesterol 4 C^{14} During the first 30 min after the cholesterol-4 C^{14} injection the decrease of radioactivity is rapid and thereupon slows down very markedly (Fig 1)

When the disappearance of the cholesterol label was followed for a longer period of time as shown in Fig 2 it was noted that the mode of disappearance was not strictly linear when plotted on semilogarithmic paper During the time interval from 25 to 145 hours an apparent turnover time of the α_1 lipoprotein cholesterol was estimated as 52 hours

Discussion

The percentage of intravenously given cholesterol-4 C^{14} in the lipoprotein fractions is about the same as the percentage of chemically determined cholesterol in the same lipoprotein fractions (Miettinen 1957) and also the same

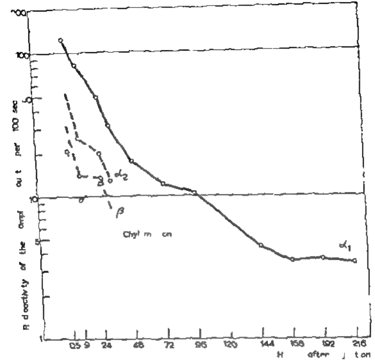

Fig 2 Semilogarithmic plot of the disappearance of radioactivity of α_1, α_2 and β lipoprotein and chylomicron fractions after intravenous injection of cholesterol-4-C[14]

when cholesterol-4-C[14] is given orally (KARVINEN and MIETTINEN to be published) Already 2 minutes after cholesterol 4 C[14] was given intravenously it was found to be distributed between the lipoprotein fractions in the same way as cholesterol according to chemical analyses is distributed and a minor residue of radioactive lipid only was found in the serum not bound in the lipoproteins Furthermore the decrease in cholesterol-4-C[14] activity in the lipoprotein fractions during the first 30 minutes is very rapid and parallel with the decrease in the residual radioactivity in the serum

These findings seem to indicate that the uptake of cholesterol-4 C[14] did not occur in the liver but was accomplished in the blood itself either within the vascular system or during the period when the blood samples taken were being prepared for electrophoretic separation In fact WHEREAT and STAPLE (1960) found that cholesterol-4 C[14] in a Tween 20 dispersion exchanges readily with the cholesterol of rat plasma lipoproteins in vitro On the basis of electrophoretic ultracentrifugal and immunochemical evidence they concluded that this was a true isotope exchange and that the physical properties of the lipoproteins were not altered Lipoproteins containing labeled cholesterol showed identical SF values when compared with isolated unlabeled lipoproteins

Acta physiol scand 1962 56 157—161

From the Institute of Occupational Health, Helsinki Finland

Effect of Ethionine on the Transport of Cholesterol-4-C^{14} in Rat Lipoproteins

By

Esko Kärvinen and Matti Miettinen

Received 27 February 1962

Abstract

Kärvinen E and M Miettinen *Effect of ethionine on the transport of cholesterol 4 C^{14} in rat lipoproteins* Acta physiol scand 1962 56 157—161 — Ethionine administration to female rats resulted in a significant increase in the radioactivity of the chylomicron fraction after ingestion of cholesterol-4 C^{14} On the other hand ethionine administration resulted in a marked decrease in the incorporation of radioactivity into the α_1 lipoprotein fraction after ingestion of cholesterol-4 C^{14} In the normal rat 70 % of the radioactivity contained in the serum was found in the lipid of α_1 lipoprotein whereas in the ethionine rats a trace of radioactivity only was found in the α_1 fraction There were no significant differences in the activities of the α_2 and β lipoprotein fractions between the ethionine and control rats It is concluded that liver damage by ethionine resulted in a specific inhibition of the formation of α_1 lipoprotein

A liver damage produced by ethionine administration is known to reduce serum cholesterol phospholipids and fatty acids (Feinberg et al 1954) and both the high and low density lipoproteins approximately to the same extent in the dog (Feinberg et al Wang et al 1958) In the rabbit a relative decrease in the α lipoprotein and an increase in the β lipoprotein – chylomicron fraction after ethionine administration has been noted (Wang et al) In man serum α lipoprotein is reported to be low in liver diseases (Eder and Russ 1952 Kunkel and Slater 1952 Nikkila 1953)

In the rat effects of ethionine on the blood lipids and lipoproteins have not been studied However it has been reported that ethionine is able to induce a fatty liver (Farber Simpson and Tarver 1950 Farber Koch Weser and Popper 1951) and to inhibit hepatic protein synthesis in the female rat (Farber and Corban 1958) Therefore it seemed worthwhile to

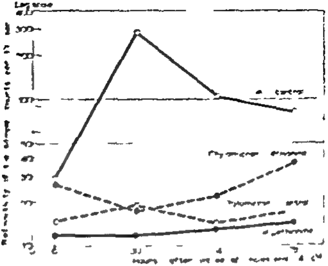

Fig. 1. Semilogarithmic plot of the radioactivity of the lipoprotein and cholesterol fractions in ethionine and control rats after intake of cholesterol-4-C^{14}.

find out what effects ethionine might have on the transport of cholesterol by the lipoproteins in this species.

Material and Methods

Female rats of the Wistar strain weighing 200 to 250 g were caged individually and given a stock diet for 3 weeks ad libitum. During the experiment the rats were fed with graham flour and water.

On the first and second experimental day the ethionine rats were given daily 3 intraperitoneal injections of 50 mg of DL-ethionine dissolved in 2 ml of saline at 9 a.m., 3 p.m. and 10 p.m. On the third and fourth day 2 i.p. injections of 50 mg of ethionine were given daily at 9 a.m. and 3 p.m. On the fifth day 50 mg of ethionine were given i.p. at 9 a.m.

The control rats were given i.p. injections of 2 ml of saline according to the same schedule.

Between the first and second day all rats were fasted overnight and fed on the second day at 9 a.m. 11 μC of cholesterol-4-C^{14} in 2 g of graham flour. The labeled cholesterol was supplied by the Radiochemical Centre, Amersham, England. Cholesterol-4-C^{14} was dissolved in ether and poured on the dry flour. Then the dry flour was made to a paste by adding water. The rats ate the paste in about an hour.

Blood samples of approximately 1 ml were taken under ether anesthesia from the tail at 3 p.m. on the second through the fifth day. Fractionation of the serum lipoproteins was carried out according to the paper electrophoretic method of [illegible] (19[illegible]3) as modified by [illegible] (19[illegible]). The electrophoresis paper was cut into stripes containing albumin plus α_1 globulin plus α_1 lipoprotein, α_2 globulin plus α_2 lipoprotein, β globulin plus β lipoprotein and the area around the starting line containing chylomicrons. The stripes were boiled in 10 [illegible] ethanolic KOH for 2 hours. The unsaponifiable lipid was taken up in petroleum ether and washed with 50 % ethanol and the with water. The unsaponifiable lipid was then transferred to steel planchets and assayed for radioactivity by using a thin mica window Geiger tube (20th Century EW 3H) and an EKCO Automatic Scaler N 530 F.

Table I Mean radioactivities contained in the unsaponifiable lipid of the lipoprotein and chylomicron fractions of the ethionine and control rats The values are unweighted means including all values from the 2nd to the 5th day expressed in counts per 100 sec per 0 5 ml of serum

Fraction	Ethionine rats determin = 11		Control rats determin = 7		Probability of difference ethionine vs. control (t test)	% of total activity	
	Mean	Stand. dev	Mean	Stand. dev		Ethionine	Control
Alpha 1	5.7	3.1	129.1	119.2	0.01	9	70
Alpha 2	12.2	7.2	23.3	17.1	None	18	13
Beta	22.1	15.2	18.7	18.9	None	34	10
Chylomicron	25.3	10.1	13.1	6.9	0.02	39	7
	3 rats		2 rats				
Liver	6 985	686	7 405	87	None	—	—

At 3 p.m. on the fifth day the animals were killed. Their livers were excised and immersed in ethanol The livers were then boiled with 10% ethanolic KOH for 2 hours The unsaponifiable lipid was taken up in petroleum ether and washed with 50% ethanol and water Samples of the unsaponifiable lipid were then plated on steel planchets and counted The counts were corrected for mass absorption.

Results

It is seen from Fig 1 that administration of ethionine to female rats led to an increase in radioactivity of the chylomicron fraction after giving cholesterol-4 C^{14} orally The radioactivity of the chylomicron fraction of the ethionine rats was significantly higher than that of the controls (Table I)

On the other hand it is seen from Fig 1 that administration of ethionine resulted in a marked decrease in the radioactivity of the α_1 lipoprotein fraction when compared with the control group The difference in the activity of the α_1 fractions between the ethionine and control group was significant (Table I) In the controls majority of the radioactivity contained in the lipoprotein lipid was found in the α_1 fraction whereas in the ethionine rats a trace of radioactivity only was found in this lipoprotein fraction

In Fig 2 the radioactivities of the α_2 and β lipoprotein fractions are shown. There were no significant differences in the activities of the α_2 and β fractions between the ethionine and control animals

At the end of the experiment, the activities remaining in the livers of the ethionine animals did not differ significantly from those of the control animals (Table I)

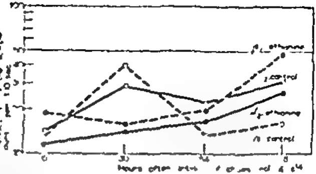

Fig. 2. Semilogarithmic plot of the radioactivities of α_1 and β_1 lipoprotein fractions in ethionine and control rats after intake of cholesterol-4-C^{14}

Discussion

The present results show that under the influence of ethionine a trace only of cholesterol-4-C^{14} activity is incorporated into α_1 lipoprotein after the ingestion of the labeled cholesterol. On the other hand in the control group a large majority of the cholesterol-4-C^{14} activity contained in the lipoproteins was found in the α_1 fraction. Similar findings with normal rats have been reported elsewhere. KARVINEN and MIETTINEN (1962) found that 60 to 70 per cent of the cholesterol-4-C^{14} activity contained in the lipoproteins was incorporated in α_1 fraction after oral administration of the cholesterol-4-C^{14} to intact male rats. Chemical analyses further indicate a similar distribution of rat lipoprotein cholesterol with α_1 fraction carrying about 60 per cent of total serum cholesterol (MIETTINEN 1957). Thus the distribution pattern in the ethionine animals departs very distinctly from the pattern normally found as to the distribution of cholesterol between the different lipoproteins in the rat.

Furthermore it was noted that the ethionine group had a significantly higher activity in the chylomicron fraction than did the control animals. This indicates that the ethionine rats had a sufficiently large supply of cholesterol-4-C^{14} to the liver in order to synthesize lipoproteins containing labeled cholesterol. On the other hand it seems likely that a liver damaged by ethionine may not be capable of transferring cholesterol from the chylomicrons to the α_1 lipoprotein. Accordingly the chylomicrons loaded with cholesterol-4-C^{14} keep on circulating in the blood stream. It has been reported by OLIVECRONA, GEORGE and BORGSTRÖM (1961) that chylomicrons labeled with palmitic acid 1-C^{14} when injected into rats with livers damaged by ethionine disappear at a rate twice as slow as in normal rats.

It was shown by FARBER and CORBAN that ethionine inhibits the hepatic synthesis of protein in the rat. Thus in the ethionine animals a condition may exist in which the protein moiety of the α_1 lipoprotein is lacking and therefore the labeled cholesterol cannot be taken up by the α_1 lipoprotein.

The livers of both groups studied had very similar levels of radioactivity so it is unlikely that the relatively low labeling of the α_1 fraction would be due to an insufficient supply of cholesterol-4-C[14] at the site of synthesis of the α_1 lipoprotein Furthermore there were no significant differences in the α_2 and β lipoprotein fractions between the ethionine and control group This may be taken to indicate that in the ethionine animals a rather normal supply of cholesterol-4 C[14] was available at the site of synthesis of the α_2 and β fractions The same was probably true with the site of synthesis of α_1 lipoprotein too

It has been implied that ethionine may facilitate fat absorption since increased chylomicron counts were noted in rats with ethionine induced fatty livers (TIDWELL 1956) As to cholesterol, no information is available on any effect of ethionine on its absorption In the present study, the radioactivity contained in the chylomicron fraction of the ethionine animals did significantly exceed that of the control rats whereas in the α_1 lipoprotein fraction the difference in radioactivity between the ethionine and control groups was in the opposite direction Thus it is evident that the chylomicrons cannot be used in ethionine animals as an indicator of absorption in any quantitative sense

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Competitive Effects of Sympathetic Control and Tissue Metabolites on Resistance and Capacitance Vessels and Capillary Filtration in Skeletal Muscle

By

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Received 5 March 19[illegible]

Abstract

Lewis, D. H. and S. Mellander. *Competitive effects of sympathetic control and tissue metabolites on resistance and capacitance vessels and capillary filtration in skeletal muscle.* Acta physiol. scand. 1962. 56. 162—188. — Reduction in regional blood flow to skeletal muscle impairs and eventually abolishes the responses of the resistance and capacitance vessels to both sympathetic vasoconstrictor nerve fibre activation and close intraarterial infusion of l-noradrenaline. The resistance response declines more rapidly and is abolished earlier than the capacitance response. The more severe the degree of flow reduction the more rapid is the decline and the shorter is the time to abolition of both responses. Increased frequency of nerve stimulation and increased dosage of noradrenaline produce greater responses and can protect reactivity temporarily against the effects of flow reduction. This protection is greater for the capacitance response than for the resistance response. Flow reduction also impairs precapillary resistance to a greater degree and more rapidly than postcapillary resistance. This interferes with and eventually abolishes the normal ability of the nerves to decrease mean capillary hydrostatic pressure and thereby to cause a net inward movement of extravascular fluid. Retention of postcapillary resistance response beyond that of precapillary results eventually in an outward movement of fluid on nerve stimulation. The data indicate that precapillary responses (precapillary resistance vessels, precapillary sphincters) are more under the influence of local dilating metabolic factors than are postcapillary responses (postcapillary resistance vessels, major capacitance vessels) which, in turn, are more dominated by extrinsic nervous factors. These observations provide a more precise understanding of the regulation of the local circulation and may aid in explaining the nature of the circulatory derangement in shock.

[1] Present address: Philadelphia General Hospital, Philadelphia, Pa., USA. Work done during the tenure of an Established Investigatorship of the American Heart Association and a Special Fellowship of the National Institutes of Health.

In a previous communication from this laboratory (MELLANDER 1960) it was shown that stimulation of the sympathetic vasoconstrictor fibres to the hind part of the cat produced simultaneously a decrease in regional blood flow and a decrease in the volume of this part The decrease in blood flow was due to the response of the resistance vessels causing the wellknown increase in vascular resistance The decrease in the volume of the hind part was found to be due to two factors The first factor was the response of the capacitance vessels causing an immediate and rapid decrease in the volume of blood present in the region The second factor was a slow but continual transfer of fluid from the extravascular space to the venous return from this region, caused by a drop in mean capillary hydrostatic pressure

The observation that vasoconstrictor nerve stimulation decreased mean capillary hydrostatic pressure provided in addition, some information on the sites of the resistance response In this experimental design in which both arterial inflow pressure and venous outflow pressure were held constant a decrease in mean capillary hydrostatic pressure could be caused only by a relatively more pronounced increase in precapillary resistance than in post capillary resistance If one considers the ratio precapillary resistance/post capillary resistance then, stimulation of all regional sympathetic vasoconstrictor fibres increased this ratio

It was further shown that all the effects noted namely the response of the resistance vessels the change in precapillary to postcapillary resistance ratio the response of the capacitance vessels and the inward filtration of extra vascular fluid were augmented with increased frequency of nerve stimulation. The frequencies employed were those found previously (FOLKOW 1952) (CELANDER 1954) to be in the range of the normal operating discharge rate of the sympathetic nervous system.

From the observations on vascular reactivity noted above quantitative information on the various vascular responses to sympathetic vasoconstrictor nerve stimulation has been obtained under the condition of 'normal nutritional flow

The present study was designed to examine these same vascular responses when nutritional flow to the hind part alone was reduced below normal This had a two-fold purpose First it was thought that by providing such a challenge to the metabolism of the tissue information could be gained on the normal competition between sympathetic nervous activation and any metabolic factors that might affect vascular reactivity Second it was felt that such a reduction in blood flow by simulating the regional situation in certain acute pathophysiological states (e g shock) might shed light on the possible alteration thereby of the various vascular responses to vasoconstrictor fibre activation.

From the many investigations that have been carried out on the subject of shock it is clear that alterations in the peripheral circulation are of importance in this syndrome However there appears to be as yet no uniform

regarding either the pathogenesis of the derangement nor even its specific nature (GREER 1961). Of interest in this regard, is the fact that virtually all of the studies have focused upon the resistance response. There is no body of data on the effect of shock on the other vascular responses of the peripheral circulation.

Some of the data from this study were discussed by FOLKOW (1962) and a preliminary full scale report has been given (MELLANDER and LEWIS 1962).

Method

Observations were made on 60 cats ranging in weight from 2.3 to 3.9 kg, anesthetized intravenously with a mixture of chloralose (not more than 50 mg/kg) and urethane (not more than 100 mg/kg). In most experiments artificial respiration was given at a level sufficient to just barely suppress spontaneous breathing. A detailed description of the experimental technique and the method of analysis of the recordings has been presented previously (MELLANDER 1960). Briefly, the hind part was completely isolated from the upper part of the animal at the level of the hips, leaving intact only the abdominal aorta, the inferior caval vein, and the lumbar sympathetic nerve trunks. The intestines were removed. Tight ligatures were placed about both ankles and the base of the tail. The vascular bed that remained represented almost entirely skeletal muscle. In a few experiments the hind part was skinned to provide an almost pure skeletal muscle preparation. In some experiments reflex adrenal medullary secretion was prevented by ligating the right adrenal gland and denervating the left.

For measurement of blood flow a drop recorder, operating an ordinate writer (CLEMENTZ and RYBERG 1949), was inserted in the inferior vena cava, which was the sole outflow from the hind part. With this recorder, the height of the ordinate is inversely related to the blood flow. To calibrate the blood flow, the return from the drop recorder was temporarily diverted into a graduated cylinder. Measurements were made immediately before each nerve stimulation and at the point of maximum response during each stimulation.

To record changes in the volume of the hind part it was enclosed in a water filled temperature regulated plethysmograph. By this means phasic changes in both regional blood volume and in regional extravascular fluid volume could be determined (see below). In a few experiments changes in regional blood volume were measured simultaneously by another technique. For this, the animal's red blood cells were labelled with radioactive chromium (Cr^{51}). Variations in regional blood volume were recorded by external monitoring of the hind part (see ÅBLAD and MELLANDER 1962).

The arterial inflow pressure to the hind part was measured from the inferior mesenteric artery. *The venous outflow pressure of the hind part* could be set at any desired level by altering the height of the drop recorder above heart level. The arterial pressure of the fore part of the animal was monitored from a carotid artery.

Regional flow reduction to the hind part was obtained by means of a screw-clamp placed around the aorta just proximal to the inferior mesenteric artery. With this clamp any desired degree of flow reduction to the hind part alone could be produced. Minor adjustments of the clamp were made to maintain a constant inflow pressure during each nerve stimulation. The lumbar sympathetic trunks were sectioned at approximately the mid abdominal level. The peripheral ends immersed in paraffin oil were stimulated with a bipolar platinum electrode at the level of the fourth lumbar ganglion.

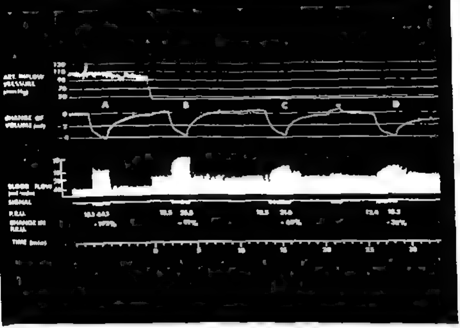


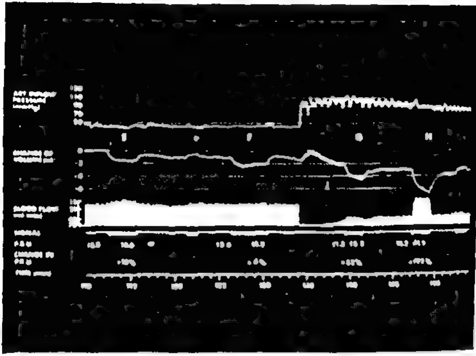
Fig 1 Skeletal muscle preparation weight 550 g Cat 3 2 kg Chloralose-urethane Effect of reduction in regional blood flow on resistance and capacitance vessels and net transcapillary filtration exchange to lumbar sympathetic vasoconstrictor nerve fibre stimulation at 4 impulses/sec (A—H) The resistance vessel responses are reflected in the changes in blood flow The values for peripheral resistance (PRU) are shown as well as the percent change in resistance caused by nerve stimulation The capacitance vessel responses are indicated by the initial rapid decrease in the volume curve and the effects on capillary filtration are indicated by the later slower and continuous changes in the volume curve during nerve stimulation Flow

At this level the sympathetic trunks contain virtually all of the efferent fibres to the hind part Supramaximal voltage (5 volts) was used to insure excitation of all fibres The duration was set at 3 msec. Various frequencies within the physiological discharge range (0—20 impulses/sec) were used Atropine was given intravenously in all experiments in a dose sufficient to block the action of the sympathetic cholinergic vasodilator fibres (LYNAS 1960)

In experiments in which the vascular responses to noradrenaline were tested this agent was administered intraarterially to the hind part through an indwelling cannula in the tail artery

With this technique phasic changes in blood flow blood volume and in net transcapillary fluid exchange induced by sympathetic vasoconstrictor nerve fibre stimulation or noradrenaline could be recorded continuously while at the same time the pressure drop across the vascular bed could be kept constant.

In each experiment the responses of the vascular bed to nerve stimulation (or noradrenaline) were tested first at the normal flow level then repeatedly during the period of reduced flow (which was kept at a constant level) and again after release of the aortic clamp



was reduced to one half of control for 134 min. The time from 33 to 110 min is not shown. Note the declining reactivity of the resistance and capacitance vessels during the period of flow reduction. During periods B through D there is a declining rate of inward movement of extravascular fluid. At E there is no net change in capillary filtration and at F there is an outward movement of fluid on nerve stimulation. With restoration of control pressure there is a partial recovery of all responses during the period of reactive hyperemia (G) and complete recovery at H. Filtration coefficient was determined at a, b and c.

Results

The results will be considered in two parts. The first part compares the reactivity of the resistance and capacitance vessels. The second part deals with the rate and direction of transcapillary filtration. From this, utilizing the concept of the filtration coefficient, the induced change in mean capillary pressure is derived, and this, in turn, is used to determine the ratio of pre-capillary to postcapillary resistance.

I. Reactivity of resistance and capacitance vessels

1. General pattern of response. Fig. 1 shows the original recording obtained from a typical experiment in which the regional lumbar sympathetic constrictor fibres were stimulated at a frequency of 1/sec for short intervals. The responses

to nerve stimulation were obtained first during the period of normal nutritional flow (control A) then repeatedly (B C, D E F) during the 134 min in which flow was reduced to approximately 50 % of the control value by partial occlusion of the abdominal aorta and finally following release of the aortic clamp (during reactive hyperemia, G and then, at control flow H) For reasons of the limitation of space the record from 33 to 110 min has been omitted However responses obtained during this period have been included in those succeeding figures derived from the experiment presented in Fig 1 The responses of the resistance vessels are indicated both by the regional resistance values (PRU) and by the percentage change in resistance The responses of the capacitance vessels and the net transcapillary filtration exchange were obtained from the volume curve As has been established previously (MELLANDER 1960) the initial rapid decline in the volume curve represents the amount of blood squeezed out of this region and indicates the response of the capacitance vessels It should be noted that the isotope monitoring method described above has confirmed this interpretation The later more slowly changing portion of the volume curve reflects changes in net capillary filtration A detailed analysis of this latter portion of the curve is given in part II of the results

From Fig 1 A (control) it can be seen that vasoconstrictor fibre activation at 4/sec increases regional vascular resistance from 15 1 to 44 1 units (mmHg/ml min/100 g tissue) and produces an initial rapid decrease in volume of 2 7 ml These responses are of about the same order of magnitude (for this size animal) as noted in earlier experiments on skeletal muscle (MELLANDER 1960) in which vascular reactivity was well maintained It can be seen from the figure (B—E) that reduction in regional blood flow is associated with a declining reactivity of both the resistance and capacitance vessels At F there is hardly any resistance response remaining while there is still a noticeable capacitance response Arterial blood pressure in the fore part of the animal remained at the control level throughout the period of flow reduction to the hind part In other similar experiments in which flow reduction was sufficiently prolonged there occurred a complete abolition of the resistance vessel response followed some time later by the disappearance of the capacitance vessel response When the aortic clamp was released at 134 min there occurred an initial period of reactive hyperemia during which partial restoration of both resistance and capacitance vessel responses was obtained (G Soon thereafter there was a restoration of control flow with nearly complete recovery of both responses (H Recovery of both vascular responses following release of the clamp indicates among other things that the nerve fibres were not damaged as a consequence of repetitive stimulation In this connection it should also be noted that with maintenance of normal nutritional flow to this region repetitive stimulations evoke at all times for several hours responses of both resistance and capacitance vessels approximately equal to the initial valu

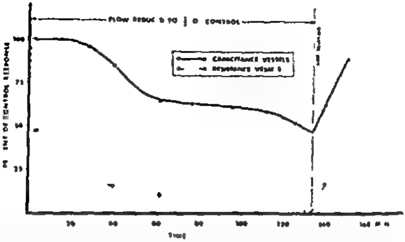


Fig. 2. Reactivity of resistance and capacitance vessels to vasoconstrictor fibre stimulation. Data taken from experiment shown in Fig. 1. Note the early and rapid decline in the resistance response which goes on to virtual abolition during the period of flow reduction (partial aortic occlusion). The decline in the capacitance response is much less pronounced. Note also recovery of both responses following release of the aortic clamp.

In Fig. 2, the responses of the resistance and capacitance vessels, as noted in Fig. 1, have been expressed as percentages of the control values. By comparing directly the reactivity of these two different vascular sections, it can be clearly seen that the time-course of the changes in reactivity is very different. There is an immediate sharp decline in the reactivity of the resistance vessels which goes on then to almost complete abolition. In contradistinction to this, the loss of reactivity of the capacitance vessels is much more gradual. Particularly striking is the rapidity of the early loss of reactivity of the resistance vessels as compared with the capacitance vessels. Thus, as can be seen in this figure, after only 5 min of flow reduction only about 40 % of the resistance response remained, while the capacitance response had not decreased at all.

Since the values obtained at the lower pressure and flow are necessarily on a different portion of the pressure-flow curve, it could at first glance be considered possible that mechanical factors might be present which could to some extent explain the rapid decline in the resistance response. This can be shown not to be the case by measuring vascular reactivity at the instant of flow reduction. In experiments in which this has been done, the results have indicated, without exception, an even somewhat greater increase in resistance response than that seen at the normal pressure, with little or no difference in the capacitance response. However, within 1—2 min, despite continued nerve stimulation, the resistance response declines markedly. The significance of this is discussed below. Viewed in this light, the control values used in this study, if anything, tend to underestimate the degree to which flow reduction

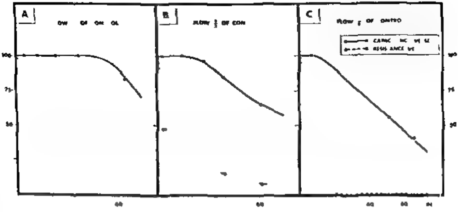


Fig 3 Effect of variations in the degree of flow reduction on the reactivity of the resistance and capacitance vessels to vasoconstrictor fibre stimulation Note that the more severe the flow reduction the more rapid is the decline in both responses At all flow levels resistance response declines more rapidly than capacitance response

interferes with the reactivity of the resistance vessels to constrictor fibre activation

Fig 2 also shows that during the period of reactive hyperemia the response of the capacitance vessels approached more nearly the control value than did the response of the resistance vessels Even so this degree of recovery of the resistance vessels was more than that usually seen In the succeeding period of normal nutritional flow both vascular responses have returned to approximately the control values

B Effect of different degrees of flow reduction Fig 3 shows the vascular responses expressed as in Fig 1 obtained in three different typical experiments in all of which the nerve fibres were excited at a rate of 4/sec The regional flow was reduced to about 3/5 of the control value in A to 1/2 in B and to 1/5 in C and in all three experiments was maintained for approximately 75 min At all three levels of flow reduction the pattern of response was similar in that there was a more rapid decline in the reactivity of the resistance vessels than in the capacitance vessels As expected when flow reduction was sufficiently prolonged the greater the degree of flow reduction the shorter was the time to disappearance of both the resistance and capacitance responses In every experiment the resistance response disappeared much earlier than the capacitance response

Table I based on 19 exp shows for 3 ranges of flow reduction the percentage of the capacitance and resistance responses remaining after 5 min and 60 min It should be stressed however that the individual animal differences are great The numbers therefore can only be considered as rough averages Furthermore individual variations should not obscure the fact that

Table 1. Effect of various degrees of flow reduction on the resistance and capacitance responses to sympathetic stimulation at 4/sec

Fraction of control flow supplying tissue	Number of experiments	Percent of control response remaining at end of			
		5 min of flow reduction		60 min of flow reduction	
		Resistance response	Capacitance response	Resistance response	Capacitance response
3/5	2	61	[illegible]	15	67
1/2	7	49	91	5	41
1/5	10	45	[illegible]	0	30

in every one of 60 exp. the resistance response declined more rapidly and failed earlier than the capacitance response.

C. Effect of different frequencies of nerve stimulation. As mentioned in the introduction it has been shown (MELLANDER 1960) that the response of both the resistance and capacitance vessels at normal nutritional flow, increases with increasing frequency of sympathetic stimulation over the physiological range, (i. e. 0—20 imp/sec). More precisely, the maximum response of the resistance vessels is reached at a rate of approximately 16 imp/sec while the maximum response of the capacitance vessels is obtained as low as 6—8 imp/sec.

The observations of this study indicated that at any frequency of vasoconstrictor nerve fibre activation in the physiological range the general pattern of response of the resistance and the capacitance vessels when regional flow was reduced was the same as that described above in section A. To provide some quantitative information a more detailed investigation has been carried out comparing, in the same animal, the responses of both vascular sections to low frequency and high frequency stimulation. The lower frequency chosen was 2 or 4 imp/sec which may represent low to moderate activity of the sympathetic system. Experiments performed at 4/sec form the bulk of the data in this report. The higher frequency chosen for comparison was 10/sec since it may be thought to represent near maximal discharge of the sympathetic system in the intact animal (FOLKOW 1955). Fig. 4 shows a typical experiment in an animal in which the responses of the resistance and capacitance vessels were obtained at both 2 and 10 imp/sec. For purposes of comparison, the responses at 10/sec have been expressed as percentages of the control values obtained at 2/sec. As expected, in the control period, both vascular responses were greater at the higher frequency than at the lower one. Comparison of the two capacitance response curves reveals that at all times during the period of flow reduction the responses at the higher frequency was greater than at the lower frequency. Furthermore at the higher frequency, it is evident that it takes a longer time to reach abolition of this response. Similarly, the resistance response at the higher frequency was greater and the time to disappearance

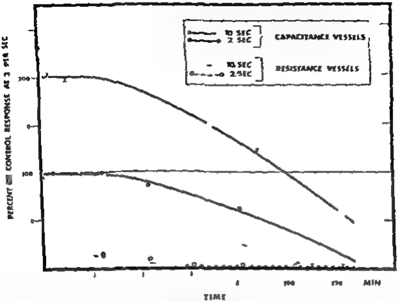


Fig 4 Effect of variations in the frequency of vasoconstrictor fibre stimulation on the reactivity of the resistance and capacitance vessels Comparison of responses to 2 and 10 imp/sec in the same animal with flow reduced to 1/2 of control The responses have been expressed as percentages of the control values obtained at 2 imp/sec The responses at 10 imp/sec are greater than at 2 imp/sec with the exception that after 108 min the resistance response is abolished at both frequencies For any given frequency the resistance response declines more rapidly than the capacitance response Note that the increased reactivity obtained at the higher frequency is more prolonged for the capacitance response than for the resistance response For example it takes approximately 100 min for the capacitance response at 10 imp/sec to decline to the control value obtained at 2 imp/sec while for the resistance response this level is reached after only 15 min of flow reduction

longer than at the lower frequency However the difference between the response at the higher frequency and that at the lower frequency was greater for the capacitance vessels than for the resistance vessels The effect of the higher frequency on the time to abolition of the two responses was different too The higher frequency prolonged the time to abolition of the capacitance response more than for the resistance response

This is not shown in Fig 4 but has been determined in other similar experiments

Two features of the curves in Fig 4 deserve emphasis First, moving to the higher frequency during the period of regional flow reduction yields greater vascular responses than can be obtained at the lower frequency with normal nutritional flow For both vascular responses this is only temporary but the length of time during which this is manifest is greater for the capacitance response than for the resistance response Second from the point of view of maintenance of the control level of reactivity seen at the lower frequency, moving to the higher frequency provides again temporarily some degree of protection of both vascular responses against the effects of regional flow re

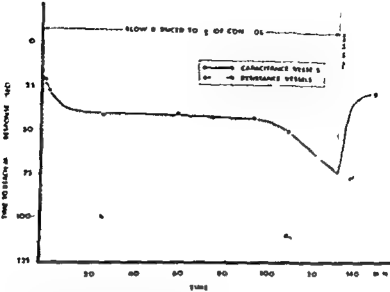


Fig. 5. Time to reach peak response during nerve stimulation (4 imp/sec). Data taken from experiment shown in Fig. 1. Note that the numbers on the ordinate are plotted from above downward. During the period of flow reduction the time to reach peak response increases for both sections but more so for the resistance vessels.

duction. Fig. 4 shows that it takes approximately 100 min of flow reduction for the capacitance response at 10 sec to fall to the control level at 2 sec. The corresponding decline in the resistance response, even though it starts at a higher level, takes no more than 15 min.

D. Time to reach peak response during nerve stimulation. Fig. 1 shows that regional flow reduction diminishes not only the absolute magnitude of both the resistance and capacitance responses but also alters the rate of development of each response during the time of nerve stimulation. In the control period nerve stimulation causes an abrupt increase in resistance which reaches maximum very quickly and then plateaus. During the period of regional flow reduction the increase in resistance on nerve stimulation is more gradual and the time to reach peak response prolonged. Similarly, for the capacitance response, it can be seen that during the period of flow reduction there is a decrease in the rate at which the capacitance vessels empty. Fig. 5, based on the same experiment as Fig. 1, shows the time to reach peak response for each nerve stimulation. During the period of flow reduction the time to reach peak response on nerve stimulation for the resistance vessels increased more rapidly than for the capacitance vessels. In the control period and soon after release of the aortic clamp the time to reach peak response was about equal for both vascular sections.

E. Effect of noradrenaline. Fig. 6 shows data from a typical experiment in which the responses of the resistance and capacitance vessels to close intra-

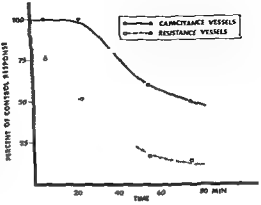


Fig 6 Reactivity of resistance and capacitance vessels to close intraarterial infusion of l noradrenaline (4μg/kg hind part/min) The pattern of response is similar to that seen with vasoconstrictor nerve fibre stimulation

arterial infusion of noradrenaline (4 μg/kg of hind part/min) were tested As can be seen the pattern of response is similar to that observed with nerve stimulation It was also demonstrated that increasing the dosage of noradrenaline but still in the physiological range, i e up to 5 μg/kg/min (Celander 1954) protected the vascular responses in the same way that increased frequency of nerve stimulation did It could also be shown that larger or 'pharmacological' doses of noradrenaline could elicit vascular responses of both the resistance and capacitance vessels at a time when neither nerve stimulation nor physiological doses of noradrenaline did

Summary

Reduction in regional blood flow to a skeletal muscle region of the cat decreases the reactivity of the resistance and capacitance vessels to both vasoconstrictor nerve fibre stimulation and to physiological doses of noradrenaline The decrease in reactivity occurs in both the absolute magnitude of the responses and in the rate of development of the responses If flow reduction is sufficiently prolonged the responses of both vascular sections will be abolished During the period of flow reduction the rate of decline of the resistance response is more rapid than the capacitance response The greater the degree of flow reduction the more rapid is the decline in both responses At any degree of flow reduction increased frequency of nerve stimulation or increased dosage of noradrenaline by producing greater vascular responses has the effect of protecting these responses temporarily against the effects of flow reduction This protective effect is greater for the capacitance response than for the resistance response. Restoration of flow to normal is associated with recovery of both vascular responses.

II Reactivity of precapillary and postcapillary resistance vessels in relation to transcapillary filtration exchange

A Effect of nerve stimulation on capillary filtration exchange and correlation with resistance and capacitance responses

As has been shown previously (Mellander 1960) and as can be seen in Fig 1 A vasoconstrictor nerve fibre activation causes a net movement

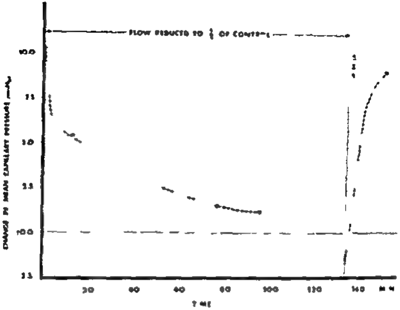


Fig. 8. Calculated change in mean capillary pressure on nerve stimulation (4 imp/sec). Data also taken from experiment shown in Fig. 1.

pressure slightly more than 10 mmHg. With regional flow reduction there was an immediate and pronounced impairment in the ability of nerve stimulation to lower mean capillary pressure. At the end of 112 min of flow reduction this ability was completely lost and 14 min later nerve stimulation increased mean capillary pressure 2 mmHg. After release of the clamp nerve stimulation was again able to decrease mean capillary pressure. Thirteen minutes after release of the clamp the value obtained was approximately the same as the control value (i.e. 9 mmHg).

It should be noted that in some experiments nerve stimulation late in the period of flow reduction was able to increase mean capillary hydrostatic pressure as much as 4 mmHg.

C. *Effect of precapillary to postcapillary resistance ratio.* The resistance vessels can be divided into two consecutive sections, a precapillary and a postcapillary one. Precapillary resistance can be defined as (mean arterial inflow pressure minus mean capillary hydrostatic pressure)/flow and postcapillary resistance as (mean capillary hydrostatic pressure minus venous outflow pressure)/flow. There is evidence to indicate that vasoconstrictor nerve fibre activation increases both pre- and postcapillary resistance (MELLANDER 1960) (BURCH and DEPASQUALE 1961). In this preparation with constant arterial inflow pressure and venous outflow pressure any change in mean capillary hydrostatic pressure induced by nerve stimulation must therefore imply a change in the ratio of precapillary to postcapillary resistance (cf. MELLANDER 1960).

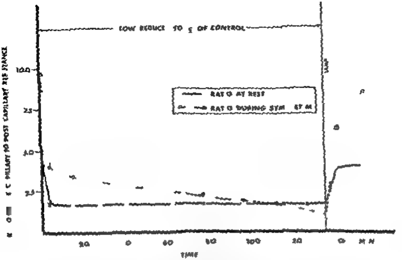


Fig 9 Ratio of precapillary resistance to postcapillary resistance Effect of flow reduction on the ratio at rest (*solid line*) and on the ability of vasoconstrictor fibre stimulation to change this ratio (dashed line)

Consideration of the precapillary to postcapillary resistance ratio affords an immediate insight into two important and interrelated factors First it gives information on the relative magnitude of these resistances Second this ratio is a major determinant of capillary hydrostatic pressure and will therefore directly affect capillary filtration exchange

In this study arterial inflow pressure venous outflow pressure and the change in mean capillary pressure on nerve stimulation were measured To calculate precisely the ratio of precapillary to postcapillary resistance one needs to know in addition the mean capillary pressure before each nerve stimulation For the purposes of this study an exact quantitation of this ratio was not deemed essential Rather interest was focused more on an approximate evaluation of the effect of flow reduction on the known ability of nerve stimulation to increase the ratio of precapillary to postcapillary resistance

Values for mean capillary pressure at rest in the control period were assumed using the data of previous workers (PAPPENHEIMER and SOTO RIVERA 1948) At the onset of flow reduction there sometimes appeared to be a change in mean capillary pressure as judged from the steady deviation of the volume curve from isovolumetric Lowering arterial inflow pressure sometimes caused a decrease in mean capillary pressure or did not change mean capillary pressure In most cases however when arterial inflow pressure was reduced there was an immediate outward filtration indicating that mean capillary pressure had increased This perhaps paradoxical finding which appears to be due to a relaxation mainly of the precapillary vessels will be discussed

more detail in a subsequent communication Of interest too, and of particular importance for this study, is the observation that not only was the new level of capillary pressure established very shortly after the institution of flow reduction but also if the degree of flow reduction was not subsequently altered then capillary pressure underwent little if any further changes

To construct the curves shown in Fig 9 the following was done The values obtained for arterial inflow pressure and venous outflow pressure from the experiment shown in Fig 1 were used with an assumed mean capillary pressure of 30 mmHg to arrive at the ratio at rest in the control period The curve of the ratio at rest (solid line) during the period of flow reduction, was obtained by using the observed arterial and venous pressures during this period with the new level of mean capillary pressure This latter value was derived from whatever changes were noted in the volume curve following the institution of flow reduction (see above) The data from Fig 8 on the effect of nerve stimulation on mean capillary pressure have been used to obtain the curve of the ratio during sympathetic stimulation (dashed line) The solid line shows that reduction in regional blood flow was associated with an almost immediate decrease in the precapillary to postcapillary resistance ratio From the dashed line it can be seen that with flow reduction there was a decline in the ability of vasoconstrictor nerve fibre activation to increase the precapillary to postcapillary resistance ratio This proceeded to the point at which nerve stimulation failed to alter the ratio (at 112 min) and beyond this nerve stimulation produced a decrease in this ratio With release of the aortic clamp the ability of nerve stimulation to increase the ratio was restored

These observations indicate that regional flow reduction has a more marked dilator effect on precapillary resistance vessels than on postcapillary resistance vessels Furthermore flow reduction interferes more with the response of the precapillary section to vasoconstrictor nerve fibre activation than with the postcapillary section

D Effect of various degrees of flow reduction frequencies of nerve stimulation and dosages of l noradrenaline Experiments have demonstrated that the more severe the regional flow reduction the smaller was the inward transfer of extravascular fluid on nerve stimulation Similarly the failure of nerve stimulation to alter net capillary filtration transfer and the immediately following phenomenon of an outward movement of fluid on nerve stimulation were reached more quickly the more severe the flow reduction From this it follows that the more severe the flow reduction the greater was the impairment of the ability of nerve stimulation to decrease mean capillary pressure and to increase the ratio of precapillary to postcapillary resistance At any given degree of flow reduction increasing the frequency of nerve stimulation by providing a more pronounced inward filtration prolonged the time during which this fluid transfer occurred The pattern of response of capillary filtration to 'physiological doses of noradrenaline was similar to that seen with nerve stimula

tion It also appeared that larger doses of noradrenaline could produce an inward movement of fluid at a time when nerve stimulation and physiological doses of noradrenaline caused an outward movement

The effect of different frequencies of nerve stimulation and dosage of noradrenaline on fluid movement during the phase of outward filtration conceivably of importance in pathophysiological states will be dealt with in more detail in a subsequent communication.

Summary

Activation of the sympathetic vasoconstrictor nerve fibres to the skeletal muscle of the cat and close intraarterial infusions of physiological doses of noradrenaline produce a more pronounced increase in precapillary resistance than in postcapillary resistance (i e increase the ratio of precapillary to postcapillary resistance) This causes a reduction in mean capillary pressure and results in a net inward movement of extravascular fluid Reduction in regional blood flow impairs the development of precapillary resistance more so than postcapillary resistance Therefore during the period of flow reduction nerve stimulation causes less of an increase in precapillary to post-capillary resistance ratio less of a decrease in mean capillary pressure and a smaller net inward capillary filtration If flow reduction is sufficiently prolonged a point is reached at which nerve stimulation does not change the ratio of precapillary to postcapillary resistance with the result that there is then no change in mean capillary pressure and no effect on the net transfer of capillary fluid Following this it can be seen that nerve stimulation will actually cause a decrease in precapillary to postcapillary resistance ratio resulting in an increase in mean capillary pressure and a net outward movement of fluid

The greater the degree of regional flow reduction the smaller is the increase in precapillary to postcapillary resistance ratio on nerve stimulation and the more rapidly is the point reached at which nerve stimulation causes an outward movement of capillary fluid At any degree of flow reduction the higher the frequency of nerve stimulation the greater is the increase in the precapillary to postcapillary resistance ratio with a consequent prolongation of the period of time during which nerve stimulation can cause a net inward movement of extravascular fluid

General discussion

1 *General considerations and aspects of normal control* The effect of sympathetic vasoconstrictor nerve fibre activation on any given vascular bed is to produce a number of responses which play separate and distinct roles in circulatory homeostasis In addition to the wellknown and extensively studied increase in resistance to blood flow there are also the equally important decrease in capacitance (volume of blood in the region) the increase in the ratio of precapillary to postcapillary resistance and with this the change in net capillary filtration transfer The principal value of the technique used in this study is that it permits a simultaneous appraisal of all these vascular responses and thus provides more detailed information than has previously been available

Reduction in blood supply is known to occur in certain vascular beds including skeletal muscle in a variety of circumstances. It occurs in the normal when there is redistribution of blood flow (e.g. changes in posture) and also in certain pathophysiological states (e.g. shock) (SAPIRSTEIN, SAPIRSTEIN and BREDEMEYER 1960; (SELKURT and ROTHE 1961). It is also known that in such circumstances there is a reflexly increased activity of the sympathetic vasoconstrictor system (e.g. NEIL 1962). Therefore it was deemed worthwhile to investigate the effect of reduction in regional blood flow on the ability of the sympathetic vasoconstrictor nerve fibres to elicit the above mentioned vascular responses in skeletal muscle.

It has been shown in this study that reduction in regional blood flow to the skeletal muscle of the cat interferes with the normal ability of the sympathetic vasoconstrictors to increase regional resistance, decrease capacitance, increase the precapillary to postcapillary resistance ratio, decrease mean capillary hydrostatic pressure and thereby cause a net inward movement of extravascular fluid.

It seems most likely that the change in vascular reactivity associated with reduction in regional blood flow is due to a relative accumulation of metabolites, as will be discussed in more detail below. It could be argued that certain other factors might be responsible. Failure of transmission of the sympathetic nerve impulse might occur, but during shock there is evidence to indicate that the sympathetic impulse traffic is not interfered with (e.g. NEIL 1962). Trauma to nerves from handling could result in failure of response to electrical stimulation. The demonstration that restoration of flow results in recovery of responses satisfactorily rules out this possibility (compare Fig. 1 A and 1 B). Failure of release of the neurohumoral transmitter substance at the adrenergic nerve endings would to some extent explain the results obtained. However, neither such a phenomenon nor any of the other above mentioned factors could explain retention of the capacitance response in the face of a markedly reduced resistance response (Fig. 2).

Since the observations made during the period of reduced regional blood flow are necessarily on a different part of the pressure flow curve than during the control period, it might at first glance be considered that the difference in responses, especially resistance, could be a purely mechanical phenomenon. This seems highly unlikely for several reasons. From a mechanical point of view, resistance responses at a reduced pressure and lowered flow should be if anything greater than at a higher pressure (FOLKOW and ÖBERG 1959). This fact was also established in the present experiments by showing that resistance responses elicited at the instant of flow reduction were somewhat greater than the control value. This means that the calculations made here, using the response at normal pressure as the control value, are conservative in the evaluation of the rapidity of decline of the resistance responses during flow reduction. It should further be noted that very shortly after the onset of flow reduction

there was instead of an increased resistance a marked decline in the resistance response to nerve stimulation Neither the above mechanical hypothesis nor any other mechanical factor occurring during the period of reduced flow will explain the progressive decline in all of the responses and their eventual abolition This is especially so since great care was taken to maintain a constant arterial pressure throughout the period of flow reduction

It is known that products of tissue metabolism have the effect of relaxing vascular smooth muscle Since reduction of blood supply to resting skeletal muscle kept at a constant temperature can be expected to result as mentioned above in a relative accumulation of metabolites, it seems logical to conclude that it is this factor which is responsible for the impaired reactivity to vasoconstrictor nerve fibre activation The exact mechanism by which the vascular smooth muscle becomes progressively less reactive and the specific agent (or agents) responsible are not known at this time The term accumulation of metabolites is used here to express a change in the environment of the vascular smooth muscle and does not deny that a deficiency of some nutrient (or nutrients) (*e g* oxygen) might be important The alteration in reactivity might be explained as a balance between the constricting effect of the nerves and the dilating action of the metabolites The metabolites may also alter the ability of the smooth muscle itself to respond to the transmitter substance The observation that reduced blood flow yields responses that are not only smaller but markedly less rapid in their development (see Fig 5) is perhaps, more in favour of the latter interpretation

This study has demonstrated that the decline in the resistance response is more rapid and that the time to abolition is shorter than for the capacitance response Further it has been shown that precapillary resistance declines more rapidly than postcapillary resistance and the time to abolition also is shorter Since the major resistance vessels are located in the precapillary section of this vascular bed (*i e* smaller arteries and arterioles) and the major capacitance vessels in the postcapillary section (mainly the veins) a general conclusion can be reached that the precapillary functions are more sensitive to the accumulation of metabolites than the postcapillary functions

From a consideration of the effect of reduced flow on the filtration coefficient a more precise idea as to the relative sensitivity to metabolites of two of the precapillary functions can be deduced There is evidence to indicate that the tone of the precapillary sphincters determines the size of the capillary bed open to flow (FOLKOW and MELLANDER 1960) (RENKIN and ROSELL 1962) Therefore changes in filtration coefficient which reflect changes in the size of the capillary bed will reflect changes in the tone of the precapillary sphincters It has been found in this study and in another one from this laboratory (COBBOLD et al 1962) that with reduction in blood supply to skeletal muscle there is an almost immediate increase in filtration coefficient Similarly CELANDER and MÅRILD (1962) have shown in newborn infants that the reflex decrease

in limb blood flow induced by lowering ambient temperature was associated with an increase in the filtration coefficient. All these data indicate that with reduction in blood flow there is a relaxation of precapillary sphincters. Since this occurs more rapidly than the decline in reactivity of the precapillary resistance vessels it would appear that the precapillary sphincters are more sensitive to the accumulation of metabolites than are the precapillary resistance vessels. COBBOLD et al. also observed that with reduced flow the reactivity of the precapillary sphincters to nerve stimulation was rapidly interfered with. The results of the present study indicate that this too occurs more rapidly than the declining reactivity of the precapillary resistance vessels and is a further point in agreement with the statement regarding the relative sensitivity to metabolites of these two sections. The major part of the precapillary resistance lies in the small arteries and arterioles. The action of the precapillary sphincters normally makes up a very minor part of precapillary resistance. Therefore in considering the section responsible for changes in precapillary resistance the action of the precapillary sphincters can be virtually neglected. From these observations it can be suggested that of the various sections of the vascular bed in skeletal muscle the section most sensitive to the action of locally produced metabolic factors is the precapillary sphincters. Next most sensitive are the precapillary resistance vessels and least sensitive the postcapillary section (i.e. postcapillary resistance vessels and major capacitance vessels). The present data do not suggest any more detailed differentiation of sensitivity within this last named section.

It could be argued that the observed differences in the rate of decline of reactivity of the various vascular sections were due to factors other than [illegible] in sensitivity to the accumulation of metabolites. For example epinephrine in small amounts is known to dilate resistance vessels with little effect on capacitance vessels (MELLANDER 1960). It is therefore theoretically possible that release of epinephrine from the adrenal medulla could interfere with the resistance response and not the capacitance response during lumbar sympathetic stimulation. This possible explanation for the results obtained was ruled out by showing that the pattern of response of the various vascular sections was exactly the same when reflex adrenal medullary secretion was eliminated. Activation of sympathetic vasodilators which act in this regard similar to epinephrine (FOLKOW, MELLANDER, ÖBERG 1961) was ruled out as a possible factor by intravenous administration of atropine just *before* control nerve stimulation in a dose sufficient to block the action of these fibres. It is possible that the effect of reduced blood flow is different in vessels in the skin than in vessels in skeletal muscle. Even though the paws and the tail were excluded in this preparation there might be sufficient skin remaining to produce the overall results seen. This possibility was ruled out by showing that in the pure muscle preparation the pattern of response was exactly the same as that in which some skin was left.

With normal blood supply, activation of sympathetic vasoconstrictor fibres causes a net inward movement of extravascular fluid This is brought about by the fact that nerve stimulation increases precapillary resistance relatively more than postcapillary resistance, with a consequent fall in mean capillary hydrostatic pressure It has been shown in this study that reduction in regional blood flow interferes with the ability of the nerves to cause an inward movement of fluid This is due as has also been shown here to the fact that reduction in regional blood flow more profoundly affects the development of precapillary resistance than postcapillary resistance It should be clear that this alteration in fluid movement is only in amount at first and not in direction However if flow reduction is maintained for a sufficiently long period of time the reactivity of the precapillary resistance vessels but not the postcapillary resistance vessels will be so compromised that nerve stimulation instead of decreasing mean capillary pressure actually raises it At this point nerve stimulation produces an outward movement of fluid which is a change in the direction of fluid movement In this case therefore there would be a loss of fluid from the circulation Such a loss might however be regained via the lymphatics with one important determinant of this being the action of the muscle pump

The change in the direction of transcapillary fluid movement on nerve stimulation also gives information on the relative duration of the precapillary and postcapillary resistance vessel response Outward movement of fluid caused by an increase in mean capillary hydrostatic pressure can only be brought about in this preparation by a relatively greater development of postcapillary resistance than precapillary Since this occurs at a time when total resistance increase on nerve stimulation is very nearly completely abolished it suggests that at this point precapillary resistance may be virtually abolished It is likely that all or nearly all of the resistance increase then resides in the postcapillary section and their reactivity is therefore preserved for a longer period of time than the precapillary resistance vessels The fact that at this time there was still a response of the capacitance section to nerve stimulation (Fig 1 F) is in further agreement with this view The capacitance function is mainly postcapillary A constriction of these vessels must imply some increase in resistance to flow and therefore in this respect they are part of the postcapillary resistance vessels All these data lead to the conclusion expressed earlier that the length of time that the reactivity of a vascular section is preserved during regional flow reduction is dependent in large part upon its location with respect to the capillary bed

It could be argued that the maintenance of postcapillary resistance might be due to factors other than retention of responsiveness to nerve stimulation *e g* sludging increased tissue pressure increased colloid osmotic pressure or alterations in blood viscosity During the relatively very short periods of nerve stimulation it seems inconceivable that changes in tissue pressure colloid

osmotic pressure or blood viscosity could occur in amounts to approach significance There is no question but that sludging might have occurred in some of the present experiments as seen by a slight progressive rise in residual flow resistance during the period of reduced flow Sludging appears to be more prominent in postcapillary vessels than in precapillary vessels (GELIN 1961) If this is the case, sludging would manifest itself as an outward movement of fluid independent of nerve stimulation This has been seen in a few experiments However, in experiments in which no signs of sludging were evident nerve stimulation did exactly the same thing as when sludging was apparent Therefore the data of this study strongly suggest that the outward filtration associated with nerve stimulation is produced by the action of the constrictors on the smooth muscles of the postcapillary resistance vessels

The data obtained in this study may possibly be applied to aid also in the *interpretation of certain physiological phenomena not directly studied in* this investigation First it might be expected that prolonged activation of sympathetic vasoconstrictors would be associated with an initial resistance response, which with the concomitant reduction in flow would tend to become less pronounced with time It would also be predicted that there might well be no significant alteration in the magnitude of the capacitance response with time Second the decrease in skeletal muscle vascular resistance with exercise suggests that despite the associated great increase in flow there may be a relative accumulation of metabolites If this is the case then there might well be a decreased reactivity to sympathetic nerve activation more pronounced for the resistance vessels than for the capacitance vessels Thus would have the effect of minimizing any tendency of nerve activation to reduce flow to this active tissue but at the same time the maintenance of an increased tone of the capacitance vessels would aid venous return This situation is perhaps similar to that seen in this study during the phase of reactive hyperemia though it is not certain that the metabolites are necessarily the same in these two circumstances With release of the aortic clamp there was a marked increase in blood flow with only a slight increase in the volume of blood present in the region It can be seen from Fig 1 G and from Fig 2 that during this period nerve activation is more effective on the capacitance vessels than on the resistance vessels A recent study from this department (KJELLMER 1967) on vascular reactivity in exercising skeletal muscle is in agreement with the thesis stated here

It is wellknown that the regulation of the local circulation is to a great extent under the influence of the locally produced metabolites The evidence from this study indicates that this can be more definitely localized to the precapillary portion of the vascular bed The manner in which the metabolites operate on the precapillary section is such as to maintain homeostasis Accumulation of metabolites tends to increase the blood supply to the region which *in turn* reduces the concentration of metabolites The action of the metabolites on the

precapillary section seems to be at least twofold First the action on the pre capillary resistance vessels has the effect of increasing the blood flow to the region Second the action on the precapillary sphincters has the effect of distributing the available blood over a greater portion of the capillary bed than is normally open In contradistinction to this the postcapillary section is less dominated by metabolites and is rather more under the control of extrinsic (*e g* nervous) influences This is also in keeping with the concept of homeostasis since the capacitance vessels play little if any role in local circulatory regulation but are more important in central circulatory regulation and especially in the maintenance of venous return Indeed were the capacitance vessels as sensitive to metabolites as the resistance vessels local circulatory regulation could conceivably be less readily manageable since the blood brought to the tissues would tend to accumulate there

It would appear that one function of the sympathetic vasoconstrictor fibres is to bring fluid into the circulation from the extravascular space by increasing *the ratio of precapillary to postcapillary resistance Since the precapillary* resistance is more influenced by accumulated metabolites, it is clear that with the accumulation of metabolites there is necessarily a tendency to impair this function of the nerves Despite this impairment the neurogenically induced increase in precapillary resistance is still greater than postcapillary, so that for a very long period of time with interference of blood supply the action of the nerves causes fluid to move into the circulation Furthermore, the increase in the filtration coefficient brought about by the relaxation of the pre capillary sphincters tends to compensate for this impairment

B Patho-physiological considerations It is suggested that the observations of this study can be in part applied to an examination of the effect of certain pathophysiological states in particular shock on the vascular reactivity in skeletal muscle One reason for this suggestion is that the levels of reduced regional blood flow studied here were chosen with a view towards encompassing the levels of skeletal muscle blood flow observed in various degrees of hemorrhagic shock (*e g* SAPIRSTEIN SAPIRSTEIN and BREDEMEYER 1960)

In this connection it should be emphasized first that the preparation used here is certainly not a shock preparation though in both cases skeletal muscle blood flow is less than normal Second the suggestions raised here will be the subject of another communication in which the effect of hemorrhagic shock on vascular reactivity in skeletal muscle has been shown to be very similar to that produced by regional flow reduction

It is known in shock that arterial blood pressure and skeletal muscle blood flow are reduced and that there is an increased activity of the sympathetic vasoconstrictors (NEIL 1962) Consider for this discussion, the effect that these alterations would have on the vasculature of the skeletal muscle and for the present assume that they are the only factors of significance A justification for this assumption is that it permits an analysis of the peripheral

circulation uninfluenced by other factors which factors, however, can be added to the analysis as they are investigated

Consider, first the situation in which the derangement is slight The reduced reactivity to sympathetic activation of the resistance vessels would tend to compensate for the reduced flow and in this, would be aided by the increased venous return provided for by the maintained capacitance response and the inward movement of extravascular fluid These latter two would be brought about by the increased activity of the sympathetics Conceivably the compensations could be sufficient to restore the flow to the point at which all vascular responses would be normal and the derangement thereby overcome

With a more serious derangement it is possible that the compensations would not be complete, but that the increased level of sympathetic activity could provide a steady compensatory influence that would effect a balance between the effects of the reduced flow and the action of the nervous system. In this case while reactivity of all regional vascular sections would be less than normal there might not be a steadily declining reactivity Furthermore any tendency toward declining vascular reactivity could be counteracted by increasing the frequency of sympathetic discharge

In the most severe state it could be considered that the compensations are inadequate and the declining vascular reactivity goes unchecked In this case, with eventual abolition of precapillary responses a turning point occurs at which nerve activation instead of providing an increase in circulating blood volume actually causes a loss of fluid Some idea as to the magnitude of such a loss may be suggested from the data obtained in this study For example from fig 7 (126 min) it can be seen that a point is reached at which with nerve stimulation fluid leaves the circulation and enters the extravascular space of the skeletal muscle at a rate of slightly more than 0 04 ml min/100 g tissue In one hour's time there would have been added 2 4 ml of fluid about each 100 g muscle a volume hardly detectable grossly as edema Yet in this period of time in an average sized human this would deplete the circulation by almost one liter of fluid The extent to which this fluid might be brought back into the circulation via the lymphatics is not known If the action of the muscle pump is impaired as it would seem to be in profound shock then the ability of the lymphatics to return this fluid to the circulation might be compromised

The change in the direction of fluid movement due to nerve activation would appear to be one critical point in the survival of the organism Up to this point the various effects of the sympathetic vasoconstrictor system on the muscle vascular bed all appear to be operating in a manner to compensate for the shock At this point however sympathetic activity actually is acting in a decompensating manner If such a train of events occurs in shock this phenomenon may offer a means of resolving the various discrepant observations on the efficacy or lack of efficacy of sympathomimetic and sympatholytic agents (for ref see SELLEY and WEIL ICER 1961) (LOEF 1962)

Another decompensatory factor expected in the later stages of shock is the loss of reactivity of the capacitance vessels With normal nutritional flow this study has demonstrated that maximal sympathetic vasoconstrictor activation to skeletal muscle will decrease the volume of the capacitance section by approximately 0 7 ml/100 g tissue Failure of this response in an average sized human could therefore account for 250—300 ml of blood which could be 'pooled in this tissue Furthermore the actual amount pooled would be greater than calculated here if there ■ any dilatation of the capacitance section beyond its basal state

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Acetate Metabolism in Isolated Epididymal Adipose Tissue from Obese-Hyperglycemic Mice of Different Ages

By

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Abstract

Hellman B S Larsson and S Westman *Acetate metabolism in isolated epididymal adipose tissue from obese hyperglycemic mice of different ages* Acta physiol scand 1962 *56* 189—198 — The utilization of ^{14}C 1 acetate was studied in isolated epididymal adipose tissue of adult mice with the American variety of the obese hyperglycemic syndrome A lower lipogenesis from acetate was found in these animals compared with their lean littermates In both types of mice there was a tendency for decreased acetate conversion with increasing age The considerable higher number of fat cells per unit wet weight in the lean littermates was not accompanied by any increase of the tissue nitrogen The results obtained emphasize the value of also relating biochemical data to the number of fat cells in a given amount of tissue

As a consequence of the current general opinion that the adipose tissue is the major site for the conversion of carbohydrate into fat it has been suggested that a metabolic anomaly of the fat cells might be the cause of the obesity in the American variety of the obese hyperglycemic syndrome in mice (Christophe *et al* 1961 a Hollifield Parson and Ayers 1960) Morphologically this type of obesity was not accompanied by any apparent increase in the number of fat cells (Hellman Taljedal and Westman 1962) Since an increased capacity of the individual fat cell for synthetizing fatty acids and neutral fat is a possible mechanism operating in the obese mice our present study has been confined to the *in vitro* metabolism of ^{14}C-1 acetate in the well defined epididymal fat pad As the manifestations of the syndrome are dependent on age mice in two different age groups have been compared

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Table II *μM acetate converted in different fractions of the epididymal adipose tissue expressed per 100 mg wet weight*

Mean values ± S E. The number of animals studied is indicated within brackets.

Animal	Age (mths)	μM acetate utilized per 100 mg wet weight				
		Carbon dioxide	Neutral fat	Fatty acids	Upper phase	Insoluble residue
AN						
(12)	5	1.92 ± 0.21	5.61 ± 0.72	0.196 ± 0.023	0.53 ± 0.03	0.18 ± 0.01
[1](11)	10	1.60 ± 0.14	4.69 ± 2.39	0.207 ± 0.090	0.35 ± 0.07	0.45 ± 0.20
AO						
(12)	5	1.38 ± 0.13	0.88 ± 0.10	0.065 ± 0.008	0.89 ± 0.08	0.22 ± 0.02
(12)	10	1.46 ± 0.19	0.46 ± 0.08	0.057 ± 0.013	0.42 ± 0.09	0.33 ± 0.04

[1] After exclusion of one animal with extremely small fat cells the figures were carbon dioxide 1.57 ± 0.16 neutral fat 2.44 ± 0.89 fatty acids 0.121 ± 0.027 upper phase 0.32 ± 0.08, and insoluble residue 0.26 ± 0.05

The tissues were washed in physiological saline to remove radioactive contamination from the medium Tissue lipids were extracted with chloroform methanol (2 1) in which the tissue fragments were homogenized The extract was purified by the "salty wash method of FOLCH LEES and STANLEY (1957) to remove the non lipid radioactive material In the two phase system obtained the neutral fat and the free fatty acids remained in the lower non polar phase while the methanol together with the water soluble compounds (e g glucose pyruvate acetate and glycerophosphate) collect in the upper water phase The upper phase was retained and after drying measured for radioactivity Separation of free fatty acids from neutral fat was done according to BORGSTRÖM (1952) The fatty acids after saponification with an ethanolic sodium hydroxide and acidification of the solution were extracted with petroleum-ether The two petroleum-ether extracts thus obtained containing either the neutral material or the free fatty acids were washed with 50 alcohol evaporated, made up to known volumes with fresh petroleum ether and mounted on lens paper-covered aluminium discs according to ENTENMAN et al (1949) After the extraction of lipids the rest (= insoluble residue e g proteins nucleoproteins nucleic acids and mucopolysaccharides) was dissolved in 0 1 N NaOH for 24 hours at 20 C and transferred to planchets for measurements of radioactivity

Results

The weights of the animals and epididymal fat pads oxygen consumption and the tissue nitrogen are shown in Table I There were no changes in body weights with age the AO mice weighing almost twice as much in both groups The weights of the epididymal fat pads in the younger AO mice were approximately doubled both as compared with the corresponding AN mice and the older AO mice While there was a slight increase in the epididymal fat with age in the AN mice ($t = 2.08$ $P \approx 0.05$) a significant reduction was noted in the AO mice ($t = 3.37$ $P < 0.01$) In two of the 10 months old AO

Table III μM acetate converted in different fractions of the epididymal adipose tissue per mg tissue nitrogen in the 5 months old animals The figures are calculated on the basis of Tables I and II

Mean values ± S E. The number of animals studied is indicated within brackets.

Animal	Age (mths)	μM acetate utilized per mg tissue nitrogen				
		Carbon dioxide	Neutral fat	Fatty acids	Upper phase	Insoluble residue
AN (11)	5	11.[illegible] ± 1.31	36.73 ± [illegible].50	1.2[illegible] ± 0.[illegible]3	3.05 ± 0.28	1.12 ± 0.11
AO ([illegible])	5	8.04 ± 0.59	6.09 ± 0.[illegible]8	0.40 ± 0.01	5.08 ± 0.3[illegible]	1.23 ± 0.07

Table IV The number of fat cells per mg wet weight as well as the μM acetate converted in different fractions of the epididymal adipose tissue per 10^5 fat cells

Mean values ± S E. The number of animals studied is indicated within brackets.

Animal	Age (mths)	Fat cells (per mg wet weight)	μM acetate utilized per 10^5 fat cells				
			Carbon dioxide	Neutral fat	Fatty acids	Upper phase	Insoluble residue
AN ([illegible])	5	5 530 ± 479	0.3[illegible] ± 0.02	1.31 ± 0.11	0.013 ± 0.008	0.11 ± 0.01	0.03[illegible] ± 0.00[illegible]
AN [a](9)	10	11 609 ± 5 718	0.[illegible] ± 0.0[illegible]	0.4[illegible] ± 0.01	0.0[illegible] ± 0.003	0.0[illegible] ± 0.0[illegible]	0.049 ± 0.011
AO (9)	5	2 53[illegible] ± 2[illegible]	0.56 ± 0.09	0.36 ± 0.0[illegible]	0.0[illegible] ± 0.001	0.38 ± 0.03	0.0[illegible]0 ± 0.007
AO (9)	10	3 435 ± 339	0.53 ± 0.07	0.18 ± 0.04	0.0[illegible] ± 0.003	0.13 ± 0.0[illegible]	0.140 ± 0.0[illegible]

[a] After exclusion of one animal with extremely small fat cells the figures were: fat cells [illegible]481 ± 1 688, carbon dioxide 0.29 ± 0.04, neutral fat 0.41 ± 0.0[illegible], fatty acids 0.0[illegible] ± 0.004, upper phase 0.0[illegible] ± 0.0[illegible] and insoluble residue 0.019 ± 0.012

the most distal parts of the epididymal fat pads were of a more yellow colour with a harder consistency

The results obtained when the epididymal adipose tissue was incubated with ^{14}C-1 acetate are shown in Tables II—IV In Table II the values have been expressed as μM acetate converted per 100 mg wet weight In the 5 months old animals there was less radioactivity in the CO_2 ($t = 2.21$ $P < 0.0$[illegible]) neutral fat ($t = 6.47$ $P < 0.001$) and fatty acids ($t = 10.96$ $P < 0.001$) in the AO mice On the other hand significant higher values were found in the AO mice for the total amount of radioactivity in the upper phase Since one of the 10 months old AN mice displayed extremely small epididymal fat cells (see below), the mean values for the AN mice in this age group have also

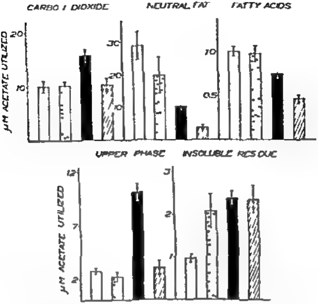

Fig 1 Acetate conversion *in vitro* by epididymal adipose tissue from 5 and 10 months old AN and AO-mice Each bar represents μM acetate converted per total epididymal adipose tissue The values are calculated on the basis of Tables 1 and 2 The lines represent the standard error of the mean ☐ = 5 months old AN mice ☐ = 10 months old AN mice ■ = 5 months old AO mice ☐ = 10 months old AO mice

been presented after exclusion of this animal Since the relative nitrogen of the epididymal fat pad is similar in 5 months old AO and AN mice the differences are virtually unchanged when the results are expressed instead per mg tissue nitrogen see Table III

The amount of acetate converted as expressed per pair of fat pads can be seen in Fig 1 Irrespective of age lower values were noted in the AO mice both for neutral fat and fatty acids the lowest incorporation being seen in the 10 months old group In the upper phase fraction a proportionally very high value was found for the 5 months old AO mice Compared with the other animals the young AN mice incorporated only about half as much exogenous acetate in the insoluble residue

Considerable differences were noted in the mean fat cell volumes both for the individual animals and also between the AN and AO mice Thus the average number of fat cells per unit weight was more than twice as high in the AN mice Since a particularly high number of fat cells in a 10 months old AN mice was associated with an extremely high rate of acetate utilization it seemed worthwhile to study the possible relation between the metabolic findings and the number of fat cells per unit weight As can be seen in Fig 2 A—D where the individual values have been plotted for the 10 months

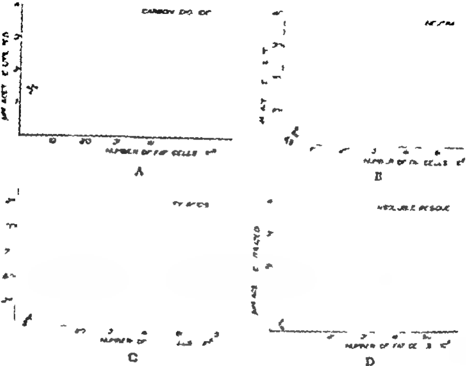

Fig. 2. The relation between the number of fat cells ($\times 10^6$) and μM acetate converted in different fractions for 10 months old animals. *A*, carbon dioxide; *B*, neutral fat; *C*, fatty acids; *D*, insoluble residue. ■ AA-mice; ○ AO-mice.

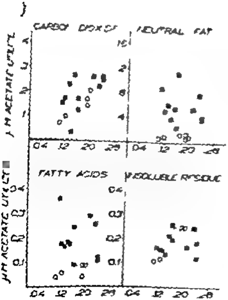

Fig. 3. The relation between the tissue nitrogen content (% of wet weight) and μM acetate converted in different fractions for the 5 months old animals. ■ = AA-mice; ○ = AO-mice.

old animals a positive correlation apparently existed at least for the neutral fat and fatty acids On the other hand there was no correlation between the acetate metabolism and the nitrogen content see Fig 3 The metabolic activity per 10 fat cells in the AN and AO mice are given in Table 4 There was also in this case a lower acetate incorporation in the neutral fat fraction for both the 5 and 10 months old AO mice ($P < 0.001$) On the other hand the formation of radioactive CO was twice as high irrespective of age in these animals Except for the insoluble residue a higher age was associated with a tendency towards decreased utilization of exogenous acetate both for the AN and AO mice Thus the value for the neutral fat in the 10 months old AN mice was less than one third of the value for the younger AN mice ($P < 0.001$)

Discussion

CHRISTOPHE JEANRENAUD MAYER and RENOLD (1961 a) recently reported that the glucose metabolism in the isolated epididymal adipose tissue was depressed in the American variety of the obese hyperglycemic syndrome in mice either when expressed on basis of unit nitrogen or wet weight On the other hand the incorporation of acetate carbon into fatty acids was increased in young animals when the tendency to obesity could first be detected (CHRISTOPHE *et al* 1961 b) Since the latter findings have placed considerable emphasis on increased lipogenesis as the factor responsible for this type of obesity, it seemed worthwhile to perform additional studies on the acetate incorporation into fatty acids and neutral fat in the epididymal fat pads from mature obese mice of two different ages

As appears from the present data no increase of *in vitro* lipogenesis was found in the adult obese animals as previously reported for mice with incipient obesity On the contrary a lower acetate utilization was noted for our AO mice both for the neutral fat and fatty acid fractions To what extent the discrepancies observed with regard to the acetate utilization in the young AO mice with developing obesity and those with a fully established obese hyperglycemic syndrome were due to different conditions of incubation is difficult to assess It is worthy of note that the conclusions of CHRISTOPHE *et al* (1961 b) are drawn essentially from experiments where no glucose was added to the medium When these authors incubated epididymal adipose tissue from six AO mice in the presence of glucose the lipogenesis was in fact equal in both obese and non obese

The marked inability of the alloxan diabetic animal to synthetize fatty acids seems to be secondary to their decreased ability to oxidize glucose While the Embden Meyerhof pathway of glycolysis is supposed to be relatively unimportant for lipid synthesis the hexosemonophosphate pathway for glucose breakdown markedly influences the rate of lipogenesis because it produces reduced triphosphopyridine nucleotide the coenzyme specifically

the formation of fatty acids (LANGDON 1957, SILVERSTEIN and FAGAN 1958 a b) It was shown with glucose labelled in the C-1 or C-6 positions that apart from the overall depression of glucose utilization the hexosemonophosphate pathway was not particularly affected in the adipose tissue of the obese hyperglycemic mice (CHRISTOPHE *et al* 1961 a) The diminished lipid synthesis observed in the present investigation is however, in agreement with the current concept of a close connection between the metabolism of lipids and glucose

The possibility of marked differences in lipogenesis of the adipose tissue at the onset of the syndrome and when it is well established is further supported by the *in vivo* observations by BATES MAYER and NAUSS (1955) These authors demonstrated that the very young obese mice retained approximately three times more ^{14}C in carcass lipids than the lean controls after administration of labelled acetate The corresponding figure for the mature obese mice was only about 30 % higher than for the AN mice It is probable that the reduced *in vitro* lipogenesis in the manifest obese hyperglycemic syndrome reflects the actual situation *in vivo* In an attempt to estimate the total daily fatty acid synthesis ZOMZELY and MAYER (1959) reported only about three times as much fatty acid synthesis from acetate in adult AO mice as in AN mice This figure is very low in view of the abundance of adipose tissue in the obese mice More than 90 % of the excess body weight in these animals was accounted for by fat the total fat content of the AO mice being 62 8 ± 1 3 % compared with 7 9 ± 0 9 % for the AN mice (BATES *et al* 1955)

The depressed labelling of the fatty acid fraction in the AO mice is not likely to be due to an increased dilution of ^{14}C-acetate since the pool of endogenous acetate has been reported to be of the same order of size in fed AO- and AN mice (ZOMZELY and MAYER 1959) It is also worthy of note that the formation of radioactive CO_2 was not correspondingly reduced In view of the complex character of the insoluble residue no conclusions can be drawn as regards the higher values found for this fraction in the AO mice The same specific defect previously found in the protein metabolism of the liver in the obese hyperglycemic syndrome (HELLMAN LARSSON and WESTMAN 1961) might also exist in the adipose tissue The tendency towards decreased acetate incorporation into CO_2 and lipids with age in both the AO and AN mice is in accord with previous findings in the rat where the adipose tissue had a significantly greater metabolic rate in young as compared with old animals (BENJAMIN *et al* 1961) Since also the adipose tissue from the AN mice was subject to metabolic changes with age this further stresses the importance of using the lean siblings as controls for studies of the AO mice

MARSHALL and ENGEL (1960) reported that during the onset phase of obesity the relative nitrogen content of adipose tissue from AO mice was only 30 % of that of AN mice With older mice the disparity tends to disappear, so that no statistically significant differences were recorded in the static phase of obesity Our observations are in essential agreement since no differ

ences were noted for the tissue nitrogen of the epididymal fat tissue in the 5 months old animals in spite of the considerable greater number of fat cells per unit weight in the AN mice (*cf* HELLMAN *et al* 1962) Because of the different number of cells i e metabolic units within a given amount of epididymal adipose tissue the metabolic activity has been expressed in terms of the number of fat cells In relating the results in this way the most prominent finding was the considerable higher oxidation of acetate in the AO mice However in both age groups the acetate incorporation into the neutral fat fraction was still lower in the AO mice as compared with the AN mice

The importance of expressing metabolic data in relation to the number of cells was evident when the individual values were plotted in diagrams Thus there seemed to be a positive correlation at least for the neutral fat and fatty acids The great individual differences found in the AN mice with regard to the number of epididymal fat cells per unit weight emphasizes the importance of considering the biochemical data with regard to the morphological findings

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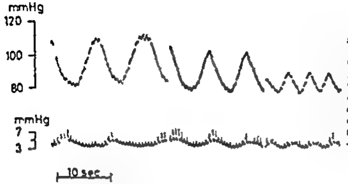

Fig 1 Arterial and right atrial pressures in a dog HLP The different parts of the tracings recorded at 3 4 and 13 min after the injection of 0.26 mg dihydro-ouabain The potassium content in plasma increased during the first 10 min after the injection from 3 90 to 4 75 meq/l

may be a purely mechanical effect, i e extension of the muscle fibres above a certain limit may lead to an increase in force of contraction followed by a gradual decrease and re extension until the previous limit is reached when an increase again takes place The importance of the rate of the extension for the generation of the waves is at present not known REITER (1962) has quite recently in experiments with stimulated papillary muscles of guinea pig heart observed mechanical after contractions (Nachkontraktionen) without any manifestation in the electrogram after administration of dihydro-ouabain He proposed an increase of the intracellular calcium as a possible cause of this phenomenon

The initiation of the present waves may however be due to a lability of myocardial function as a result of the loss of potassium in the myocardium implying that in the provoked unstable state a force development in the contractile apparatus is sensitive to changes in the fiber length This might be due to a relation between the potassium transport over the cell membrane and the extension degree of the muscle cell Thus the cellular potassium environment might be one factor determining myocardial contractile force MELVILLE and KOROL (1958) suggested in their study of the role of potassium in the stimulation and depression of the myocardial function that potassium could in some way exercise a trigger effect on the contractile mechanism The disappearance of such a possible 'trigger effect with a myocardial potassium loss below a certain critical level might provoke the automatic self oscillating and probably mechanical regulation of myocardial function described here

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Acta physiol scand 1962 56 201—219

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Effects from the Pyramidal Tract on Spinal Reflex Arcs

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Abstract

LUNDBERG A and P VOORHOEVE *Effects from the pyramidal tract on spinal reflex arcs* Acta physiol scand 1962 *56* 201—219 — The effect of stimulation of the sensorimotor cortex on spinal reflex arcs was investigated in cats with intracellular recording from motoneurones and with conditioning of monosynaptic reflexes Stimulation of cortex enhances the following synaptic actions in motoneurones Ia inhibitory Ib reciprocal, FRA reciprocal FRA inhibitory to flexor and cutaneous excitatory to extensor motoneurones The effects are mediated by the pyramidal tract which presumably exerts an excitatory action on interneurones of reflex arcs Stronger stimulation of cortex evokes synaptic actions in motoneurones with excitatory action dominating in flexor and inhibitory in extensor motoneurones There was frequently evidence of mixed excitatory and inhibitory effects in extensor motoneurones and inhibitory action in flexor motoneurones was also found It is suggested that these actions on motoneurones are due mainly to activation of interneurones of spinal reflex arcs

In cat the pyramidal tract exerts actions on alpha motoneurones via interneurones and has no monosynaptic connections with alpha motoneurones (LLOYD 1941 SZENTÁGOTHAI SCHIMERT 1941 BERNHARD and BOHM 1954 CHAMBERS and LIU 1957 HERN PHILLIPS and PORTER 1961) LLOYD (1941) demonstrated that the segmental three neurone arc reflex discharge is facilitated at an interneuronal level by volleys in the pyramidal tract In this paper it will be shown that activity in the pyramidal tract enhances the synaptic actions (excitatory and inhibitory) evoked from different classes of primary

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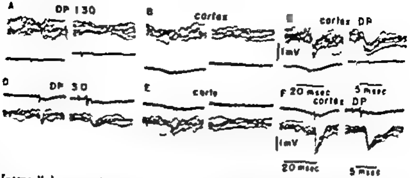

Fig. 1. Intracellular recording, internal positivity (depolarization) being signalled upwards, with microelectrodes filled with 3M KCl from a gastrocnemius-soleus (A—C) and from a plantaris (D—F) motoneurone. The left and right traces in each record were taken simultaneously at different speeds. Lower traces in A—C and upper traces in D—F were recorded from the L6 dorsal root entry zone. In A, C, D and F are shown the responses evoked by stimulation of the deep peroneal nerve at 1.30 (A and C), maximal for group Ia, and 3.0 times threshold (D and F) respectively. B and E were obtained on stimulation of the sensorimotor cortex with trains of 6 stimuli. In C and F combined stimulation of cortex and the deep peroneal nerve was used.

afferents and it will be inferred that this is due to an excitatory action on interneurones of spinal reflex arcs. A preliminary report of some of the findings has been given (LUNDBERG and VOORHOEVE 1961).

Methods

The experiments were made on 20 cats under light anaesthesia from pentobarbitalium. A large number of hindlimb nerves and in some experiments also forelimb nerves were dissected. The sensorimotor cortex was exposed on the contralateral side and the spinal cord was exposed in the lower lumbar segments. Section of the pyramid was made after exposure of the medulla from the ventral side. In most of the experiments Flaxedil was given and the animals were artificially respirated. In 13 of the experiments the synaptic actions were measured by their action on monosynaptic reflexes recorded in the ventral roots (hindlimb) or in the muscle nerves (forelimb). In 7 experiments intracellular recording was made from motoneurones with electrodes filled with 3M KCl or 0.6 M K_2SO_4 solution. Graded conditioning stimulation was used to differentiate between different afferent systems (ECCLES, ECCLES and LUNDBERG 1957; ECCLES and LUNDBERG 1959a). The stimulus strength is expressed in multiples of threshold strength.

Cortical stimulation was performed with a movable ball electrode (usually cathode but anode in one of the experiments with stimulation of the forelimb area, cf. LIVINGSTON and PHILLIPS 1957 and HERN et al. 1961), the indifferent electrode being placed in the temporal muscle. When not otherwise mentioned the ball electrode was placed in the centre of the hatched area in the postsigmoid gyrus in Fig. 3. Square wave pulses with a duration of 0.1—0.5 msec were used and the current needed to evoke liminal actions in motoneurones was usually about 0.5 mA. For investigation of actions on motoneurones currents up to 1.5 mA were used.

The lesions made were controlled in unstained frozen sections. In all figures the records consist of many superimposed traces.

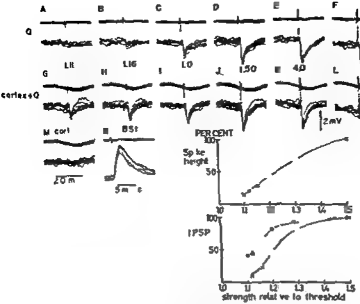

Fig 2 Intracellular recording (upper traces) with a microelectrode filled with 3M KCl from a motoneurone of the posterior biceps-semitendinosus group (BSt) Upper traces are triphasic recordings from the L6 dorsal root entry zone A F are responses evoked by stimulation of the quadriceps nerve (Q) at the indicated stimulus strengths expressed as multiples of threshold for the Q nerve In the corresponding records G—L the same stimuli are preceded by a train of 6 stimuli to the sensorimotor cortex M shows the effect of cortical stimulation alone N gives the EPSP evoked by stimulation of the posterior biceps semitendinosus nerve The insets give the size of incoming volley from Q as a function of stimulus strength (upper graph) The lower graph shows the height of the IPSP in relation to stimulus strength without (×) and with (O) conditioning stimulation from cortex

Results

1) *Ia actions*

It was regularly observed that the inhibition exerted by weak group I volleys on the monosynaptic reflex discharges from antagonist muscle nerves could be enhanced by stimulation of the sensorimotor cortex (*cf* LUNDBERG and VOORHOEVE 1961 Fig 1 Fig 5 below) The conditioning stimulus strength was always chosen so that it was subthreshold for evoking monosynaptic discharges and maximal Ia volleys were used only when they failed to evoke a monosynaptic reflex discharge Hence the facilitation from cortex must be exerted on the Ia inhibitory pathway and this was confirmed in experiments with intracellular recording from motoneurones (Fig 1 and 2) The effect was found on the Ia inhibition to extensor and flexor motoneurones of knee and ankle muscles but was most easily demonstrable on the Ia action from the ankle flexor nerves to ankle extensor motoneurones This Ia action is often absent or weak (ECCLES and LUNDBERG 1959 a) but appears when preceded by cortical stimulation (Fig 1) Record A shows the effect of a

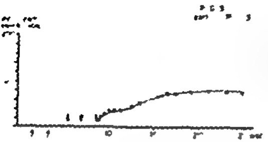

Fig. 3. The effect of cortical stimulation of the reciprocal Ia inhibition from the deep peroneal (DP) nerve on gastrocnemius-soleus (G-S) monosynaptic test reflexes. The conditioning volley from DP was evoked at a strength of 1.4 times threshold and on its entry to the cord preceded the G-S volley by 1 msec. The effect of 3 stimuli to the sensorimotor cortex is shown by circles (○) whereas crosses ( ) show the inhibition without cortical stimulation. Intervals on the abscissa are between the first cortical stimulus and the stimulated monosynaptic reflex.

Fig. 4. Drawing showing the cortical areas from which enhancement was evoked on reciprocal Ia inhibition of monosynaptic reflex discharges in hindlimb (medial pericruciate area in the postcruciate gyrus) and in forelimb (the lateral area in the coronal gyrus). The action tested in hindlimb was from deep peroneal to gastrocnemius-soleus and in forelimb from biceps brachii to caput laterale and mediale. In the hindlimb the monosynaptic reflex discharge was recorded from the ventral root. In the forelimb recording was made from the muscle nerve and the monosynaptic reflex was evoked by weak stimulation of the same nerve.

maximal Ia deep peroneal (DP) volley, B the effect of a train of 6 stimuli to the sensorimotor cortex and record C the Ia IPSP evoked by the Ia DP volley when conditioned by cortical stimulation. In some ankle extensor cells a small Ia IPSP was found even without cortical stimulation as in the plantaris (Pl) cell of D–F, Fig. 1. When the Ia volley from DP was preceded by cortical stimulation in F a much larger Ia IPSP was evoked than in D.

In motoneurones of knee flexors and extensors and of ankle flexors cortical stimulation did not usually increase the size of maximal Ia IPSP but there was almost invariably an effect on IPSPs evoked by submaximal Ia volleys. The records in Fig. 2 were taken from a motoneurone of the posterior biceps-semitendinosus (PBSt) nucleus and show the effect of volleys in the quadriceps (Q) nerve evoked at the strengths indicated between corresponding records of the upper and middle rows. The Ia volley was maximal at the stimulus strength of 1.5 times threshold. The IPSPs in G–L were obtained with cortical stimulation and in comparison with corresponding records in the upper row it is clear that there is a large increment in the submaximal IPSPs. The effect is summarized in the lower of the two graphs in the figure and the upper graph shows the relation between stimulus strength and the size of the Ia volley. The facilitatory action on the Ia IPSP in Figs. 1 and 2 can be accounted for if it is assumed that stimulation of cortex evokes excitatory action in the Ia inhibitory interneurones and hence facilitates transmission from the primary afferents to these interneurones.

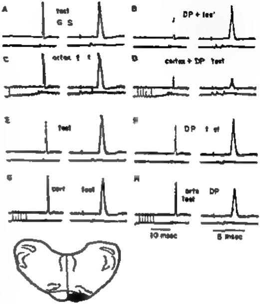

Fig 5 Simultaneous recording at two different sweep speeds in the S ventral root of the monosynaptic reflex discharge evoked by a double volley (in A—D) from the gastrocnemius-soleus (G S) nerve (upper traces) and from the L_7 dorsal root entry zone (lower traces) A shows the unconditioned test reflex and B the effect of a conditioning maximal group Ia volley in the deep peroneal nerve The corresponding records in C and D show the effect of a train of 6 stimuli to the sensorimotor cortex Cortical stimulation decreased the height of the test reflex from G S (not shown in the Fig) and to evoke the reflex in C and D the strength of the first volley from G S was increased as appears in the triphasic recordings In E—H the same series was repeated following transection of the contralateral pyramid (drawing) After pyramidal section single volleys were sufficient to evoke the G S monosynaptic test reflexes

Fig 3 shows the time course of the facilitatory action from cortex on the reciprocal Ia inhibition from the DP nerve of the G S monosynaptic reflex Enhancement of inhibition occurs in 8—10 msec after the first conditioning stimulus This latency agrees well with the latency of about 9 msec found by Lloyd (1941) for the onset of facilitation evoked by stimulation of the pyramid in the medulla on three neurone arc reflex discharges

Fig 4 shows the cortical areas from which the facilitation of reciprocal Ia inhibition of monosynaptic reflexes was obtained at slightly suprathreshold stimulation of cortex Action on Ia IPSP in motor nuclei of the hindlimb was evoked from the medial hatched area in the postsigmoid gyrus corresponding effects in the forelimb from the more lateral area in the coronal gyrus With stronger stimuli effects could be evoked from much larger cortical areas and this was also found for weak stimuli if the anaesthesia was light Cortical effects on the monosynaptic test reflexes were investigated in the same experiments and were always evoked from the same areas

The facilitatory effect from cortex on the Ia inhibitory pathway is mediated by the pyramidal tract as is proven by the finding that the action disappears after section of the pyramid (Fig 5) On the other hand after a section through the brain stem sparing the contralateral pyramid (as illustrated in Fig 11 for actions evoked from group II afferents) the effect on the reciprocal Ia inhibition remained Attention was also given to the problem of whether cortical stimulation could interfere with the monosynaptic Ia excitatory path to

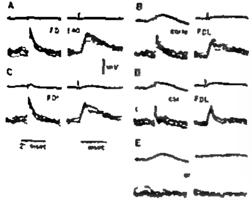

Fig 6 Intracellular recording with a microelectrode filled with 3 M KCl from a plantaris motoneurone The left and right traces in each record were taken simultaneously at different speeds The nerve to flexor digitorum longus (FDL) was stimulated in A D in A and B at a strength of 1 4 times threshold in order to evoke a maximal Ia EPSP in C and D at 1 74 times threshold which was maximal for group I Stimulation of the sensorimotor cortex is shown in E B and D were obtained with combined stimulations of cortex and the FDL nerve The upper traces were recorded from the L_7 dorsal root entry zone

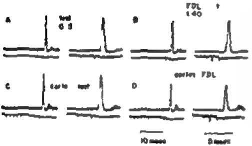

Fig 7 The effect of cortical stimulation on the inhibitory action exerted by a group I volley from flexor digitorum longus (FDL) on the monosynaptic reflex recorded from gastrocnemius-soleus (G-S) recorded simultaneously at two sweep speeds A shows the unconditioned test reflex from G S B the effect of a conditioning volley in the nerve to FDL evoked at a strength of 1 40 times threshold In the corresponding records C and D there was in addition a train of stimuli given to the sensorimotor cortex

toneurones With intracellular recording the effect of cortical stimulation investigated on the Ia EPSP in motoneurones Only in one cell of 22 estigated were we able to detect a clear cut effect as is illustrated in Fig 6 he records were obtained from a plantaris motor and show the heter onymous effect from FDL Cortical did a postsynaptic potential (F) but reduced the size B The effect in Fig 6 could be accounted for excitatory action in interneurones mediating group Ia fibres from group I fibre of fl We were however not able to in about 15 motoneur

2) *Ib actions*

When employing the it is more difficult to anal Ia pathways The reason f may influence transmission to alpha motoneurones (H group I volley usually

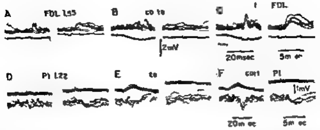

Fig 8 Intracellular recording with microelectrodes filled with 3M KCl from two gastrocnemius soleus motoneurones (A—C and D—E) The nerve to flexor digitorum longus (FDL) was stimulated in A and C at a strength of 1 55 times threshold slightly submaximal for group I The sensorimotor cortex was stimulated in B and in C in combination with the FDL nerve The PSPs in A and C are reversed IPSPs The IPSPs in D and F were evoked on stimulation of the plantaris (Pl) nerve F in combination with cortical stimulation The effect of cortical stimulation alone is shown in E The lower traces in A—C and the upper traces in D—F were recorded from the L_6 dorsal root entry zone

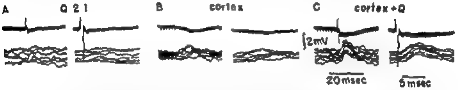

Fig 9 Intracellular recording at two sweep speeds (lower traces) from a deep peroneal motoneurone with a microelectrode filled with 3M KCl The lower traces were recorded from the L dorsal root entry zone The quadriceps nerve was stimulated at a strength of 2 1 times threshold in A and C B shows the effect of a train of 6 stimuli applied to the sensorimotor cortex and C of combined stimulation of cortex and Q

inhibition could result if the Renshaw inhibition evoked by the monosynaptic rexflex discharge was enhanced by cortical stimulation However this source of error was avoided in some experiments in which single conditioning volleys failed to evoke monosynaptic reflexes This was the case in the experiment of Fig 7 (record A) The test reflex (evoked by a double volley) was from the gastrocnemius soleus nerve (G-S) and when conditioned with the slightly submaximal group I volley from flexor digitorum longus (FDL) (the interosseus nerve removed) there was inhibition to 93 % (A B) After stimulation of cortex there was inhibition to 70 % (C D) In all likelihood the effect in D is due to cortical facilitation of Ib inhibitory interneurones Further evidence of cortical effects on Ib actions was found in experiments with intracellular recording from motoneurones In Fig 8 already been referred to above record C shows the unconditioned heteronymous EPSP in response to a maximal group I volley from FDL In D Fig 6 following cortical stimulation the decay of the PSP is much faster than in C Presumably this is due to a Ib IPSP evoked after a latency of 1 3 msec which is superimposed on the Ia EPSP The cortical effect on the Ia EPSP is shown in C and there is no change in its time course

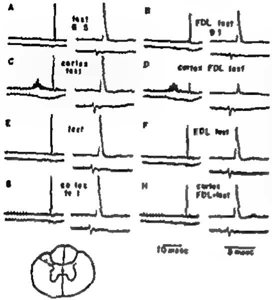

Fig 10 Simultaneous recording at two different speeds of the monosynaptic test reflex from gastrocnemius-soleus (G-S) A shows the unconditioned test reflex and B the conditioning effect of a volley from the nerve to flexor digitorum longus (FDL) evoked at a stimulus strength of 9 1 times threshold At this interval a FDL group I volley had no effect. The corresponding records in C and D show the effect of a train of 6 cortical stimuli Cortical stimulation decreased the test reflex from G S (not shown in Fig) and the test reflex in C and D was evoked by a maximal single group I volley whereas submaximal stimulation of G S was used in A and B In E—H the same series was repeated following a lesion in the ipsilateral dorsal part of the lateral funicle as shown in the drawing

compared with A Records A C Fig 8 were obtained from a G S motoneurone A and D show reversed IPSPs evoked by a slightly submaximal group I volley from FDL The records in D F are likewise from a G S motoneurone and the inhibitory potential in D and I were evoked on submaximal group I stimulation the plantaris nerve The IPSP has a latency of 1 4 msec and must be attrib- d to Ib fibres A similar facilitation was found for Ib inhibitory action voked from the nerves to C S and Q

There was also occasionally evidence of facilitatory effects on Ib excitatory actions This is illustrated in Fig 9 obtained intracellularly from a DP motoneurone An almost maximal group I volley from quadriceps has no action in A but when supported by a train of 6 cortical stimuli the same volley evoked an EPSP (central latency 1 9 msec) which can be ascribed to Ib fibres (*cf* ECCLES *et al* 1957)

Ib actions are difficult to analyse in the intact anaesthetized animal because Ib actions in this preparation are effectively suppressed (HOLMQVIST and LUNDBERG 1959) In none of the experiments in which pyramidal section was made were any Ib actions uncovered by a cortical stimulation Though we have not been able to prove by this technique that the effect illustrated in Fig 7—9 is mediated by the pyramidal tract there can however be little doubt that this is the case since it was evoked from the same cortical areas illustrated in Fig 4 and the strength of cortical stimulation required was the same as that needed to facilitate Ia inhibition and the reflex actions described in the next section

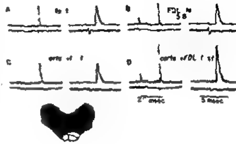

Fig 11 Simultaneous recording at two different speeds of the monosynaptic reflex evoked from the posterior biceps semitendinosus nerve BSt (upper traces) The experiment was made on a cat having the brain stems transected as shown in drawing in the mid medullary region A shows the unconditioned test reflex B the effect of a conditioning volley in the nerve to flexor digitorum longus (FDL) evoked at a strength of 5 8 times threshold The corresponding records in C and D show the effect of cortical stimulation Cortical stimulation increased the size of the test reflex of A and to obtain the test reflex and in C and D the BSt volley was decreased *cf* size of group I volley in A and C

3 *Actions from the flexor reflex afferents*

The flexor reflex afferents (FRA) consist of group II and III muscle afferents cutaneous afferents and high threshold joint afferents (*cf* ECCLES and LUNDBERG 1959 HOLMQVIST LUNDBERG and OSCARSSON 1960, HOLMQVIST and LUNDBERG 1961) Experiments were made on the effects from the FRA both with usage of the monosynaptic test method and with intracellular recording from motoneurones Fig 10 shows the small inhibition of the test reflex from the nerve to G S (shown alone in A) of a conditioning volley in group I and II afferents from FDL The inhibitory action was evoked from high threshold afferents since it appeared when the strength was raised above group I When preceded by cortical stimulation much larger inhibition was obtained (C and D) but there was also a discharge on cortical stimulation This discharge which is almost identical in C and D (slow left records) must have given activation of Renshaw interneurones and inhibition in D could be due either to facilitation of interneurones in the path from FDL to motoneurones or of interneurones in the Renshaw inhibitory pathway In other experiments the increment in inhibition was obtained without any ventral root discharge being caused by the combined conditioning of cortex and high threshold muscle afferents as has already been illustrated in Fig 7 for Ib actions Records E-H Fig 10 show that cortical stimulation of the same strength has no action after a lesion in the dorsal part of the lateral funicle interrupting the pyramidal tract It was also shown that the cortical effect disappeared after section of the contralateral pyramid as has been illustrated for Ia effects in Fig 8

Cortical effects were investigated on the actions from high threshold muscle afferents high threshold joint afferents and cutaneous afferents on monosynaptic test reflexes from extensors and flexors Enhancement of inhibition from all these afferent systems of test reflexes to extensors was found in almost all of the experiments Effects from the FRA on monosynaptic test reflexes from flexor nerves were however much more uncertain. Small effects were obtained but not regularly The explanation for this may be that volleys in

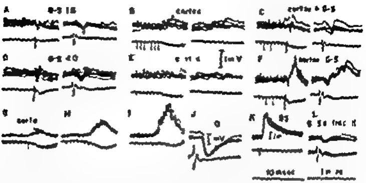

Fig. 12. Intracellular recordings (upper traces) at two speeds with a microelectrode filled with 3M KCl from a motoneurone of the posterior biceps-semitendinosus group. The lower traces were recorded at the L_7 dorsal root entry zone. The nerve to gastrocnemius-soleus (G-S) was stimulated in A and C at a strength of 1.6 times threshold and in D and F at 4.0 times threshold. B and E show the effect of cortical stimulation alone and C and F of combined stimulation of cortex and G-S nerve. G—I show the effect of stronger stimulation of the sensorimotor cortex. J, obtained afterwards, shows the Ia IPSP from Q and K the maximal Ia EPSP from BSt. The record in L was obtained after withdrawal from the cell. The calibration in E is common for A—F and L and the calibration in J for G—J.

the FRA may evoke excitatory and inhibitory action in flexor motoneurones and that both actions may be facilitated from cortex (*cf.* below). Another factor may be that the suppression of the flexor reflex arc which is most marked in the decerebrate state is also found in the anaesthetized animal with intact brain (HOLMQVIST and LUNDBERG 1959). A section of the brain stem sparing the pyramids gives large release of facilitatory action to flexor and inhibitory action to extensor motoneurones so that an animal with this lesion virtually is spinal with respect to the transmittability of the flexor reflex arc. Two experiments were made on animals with this kind of lesions and in both of them cortical stimulation gave rise to a markedly augmented facilitation from the FRA on flexor monosynaptic test reflexes (Fig. 11). There was likewise in these preparations inhibition from the FRA of monosynaptic reflexes from extensors.

With intracellular recording it was confirmed that cortical stimulation very often augmented excitatory and inhibitory post-synaptic potentials evoked from the FRA. The records in Fig. 12—17 were taken at the optimal interval to display the effect. It should be noted that in no case was the facilitation a rebound phenomenon to a preceding depression. Fig. 12 shows the effect on the EPSPs evoked by volleys in the G-S nerve in a BSt motoneurone. The cell was located at the margin of the G-S nucleus and the initial negative potential (downwards deflection) in A, C, D and F is the Ia focal field potential which is shown in the extracellular record in L. In A and C obtained at slightly submaximal group I strength there is evidence of a Ib EPSP with an onset (shown by arrow) after a latency of 1.8 msec. At 4.0 times threshold there is

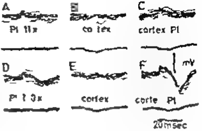

Fig 13 Intracellular recording (upper traces) from a gastrocnemius-soleus motor eurone with a micro-electrode filled with 0 6 M K SO Lower traces are triphasic recordings from the L_7 dorsal root entry zone The plantaris nerve was stimulated at 11 times threshold in A and C and at 120 times threshold in D and F B and E show the effect of cortical stimulat on alon and C and F of combined stimulation of cortex and the plantaris nerv

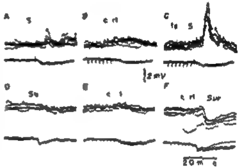

Fig 14 Intracellular record ng with microelectrodes filled with 3M KCl from a posterior b ceps-semitendinosus motoneurone in A—C and from a gastrocnemius soleus motor eurone in D—F The sural (Sur) nerve was stimulated in A and D B and E show the effect of cortical stimulation alone and C and F of combined stimulation of cortex and the sural nerve at the strength that was used to obtain the corresponding records in A and D Lower traces are triphasic recordings from the L_7 dorsal root entry zone

hardly any late EPSP in D but when conditioned by cortical stimulation (F) the G S volley evokes a large EPSP (latency 3 6 msec) which can be attributed to group II fibres Hence interneurones mediating group II excitatory action to flexor motoneurones can be facilitated from cortex Record G H and I show the excitatory action evoked in this motoneurone by cortical stimulation at increasing strength Record J was taken afterwards and shows that the reciprocal Ia IPSP from the Q nerve was still in the hyperpolarizing direction Facilitation of group III excitatory actions to flexor motoneurones was also common and is illustrated in Fig 2 record L

There was likewise enhancement of the inhibitory actions evoked by group II and III volleys in extensor motoneurones Fig 13 shows the effect of volleys in the plantaris (Pl) nerve on a G S motoneurone At the stimulus strength of 11 times threshold neither the unconditioned (A) nor the conditioned (C) Pl volley evoked an IPSP A Pl volley evoked at 120 times threshold gave a small late IPSP in D which was increased when conditioned by a train of cortical stimuli (F)

Enhancement of the reciprocal synaptic actions evoked by cutaneous volleys is shown in Fig 14 Records A C were obtained from a BSt motoneurone The sural nerve was stimulated in A and C at a strength that was just above threshold for action in this motoneurone when tested without cortical stimulation C shows the increment in the EPSP on cortical conditioning Records D F are

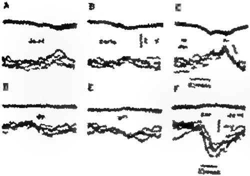

Fig. 15. Intracellular recording with microelectrodes filled with 3M KCl from a deep peroneal motoneurone in A—C and from a gastrocnemius-soleus motoneurone in D—F. The posterior nerve to the knee joint (joint) was stimulated in A, C, D and F. B and E show the effect of cortical stimulation alone and C and F of combined stimulation of cortex and the joint nerve. Upper traces were recorded from the dorsal root entry zone.

Fig. 16. Intracellular recording from a deep peroneal motoneurone with a microelectrode filled with 3M KCl. The posterior nerve to the knee joint was stimulated at the same strength in A and C. B shows the effect of stimulation of the sensorimotor cortex with a train of 6 stimuli and C combined stimulation of cortex and joint nerve. Upper traces are triphasic recordings from the L. dorsal root entry zone.

from a G-S motoneurone and reveal a corresponding facilitation of the inhibitory action.

The reciprocal flexion reflex actions can also be evoked from volleys in high threshold joint afferents (Eccles and Lundberg 1959 a) and also these actions were facilitated from cortex as is illustrated in Fig. 15.

Apart from the reciprocal actions discussed above volleys in the FRA can evoke inhibitory actions in ipsilateral flexor motoneurones (Eccles and Lundberg 1959 a, Holmqvist and Lundberg 1961). We have occasionally observed also that inhibitory actions from the FRA can be enhanced after cortical stimulation. This is illustrated for the effect from the joint nerve in Fig. 16 but was found also for actions from high threshold muscle afferents and cutaneous afferents. Investigation of the cortical effect on these inhibitory actions in flexor motoneurones was, however, difficult because of the facilitatory effect from cortex on the excitatory path from the FRA to flexor motoneurones (cf. record E, Fig. 2). For this reason we are not in the position to state if cortical stimulation facilitates the inhibitory path from the FRA to flexor motoneurones to the same extent as the other paths from the FRA to ipsilateral motoneurones.

Impulses in cutaneous afferents can evoke excitatory action in ipsilateral extensor motor nuclei (Hagbarth 1952) and with intracellular recording it was observed that also these actions could be enhanced by stimulation of the sensorimotor cortex. This is illustrated in Fig. 17 with recording from a G-S motoneurone and stimulation of the sural nerve.

In all the figures in sections 1—3 the effects from cortex are illustrated at optimal intervals between cortical and peripheral stimulation. In all instances

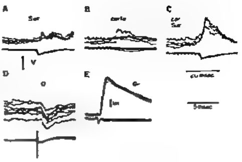

Fig 17 Intracellular recording with a microelectrode filled with 3M KCl from a gastrocnemius-soleus (G S) motoneurone The sural nerve was stimulated at the same strength in A and C. B shows the effect of stimulation of the sensorimotor cortex alone and C the effect of combined stimulation of cortex and the sural nerve Records A—D were taken during passage of a depolarizing current (4×10^{-9}) in order to prevent reversal of the IPSPs and the effect from Q taken after records A—C shows that the IPSPs were in hyperpolarizing direction Record E shows the maximal Ia EPSP from the G-S nerve Lower traces were obtained from the L_7 dorsal root entry zone

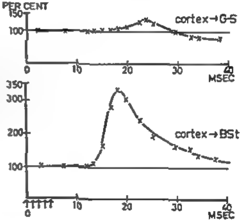

Fig 18 The effect of cortical stimulation (5 stimuli shown by arrows) on the monosynaptic test reflex discharge from gastrocnemius-soleus (G S) in the upper graph and in the lower from posterior biceps-semitendinosus In each instance 100 on the ordinates represents the unconditioned amplitude of the test monosynaptic reflex Conditioned amplitude of test reflex, expressed as percentage of control amplitude is plotted as a function of time interval between the first cortical stimulus and the monosynaptic test reflex The two curves were obtained in the same experiment with the same strength of cortical stimulation

it was carefully determined that the effect from cortex was facilitatory from the onset and that the action illustrated was not a rebound facilitation to a preceding inhibitory effect

4) *Effects on alpha motoneurones*

The primary aim of this investigation was to examine the action on the path from the primary afferents to the motoneurones but some observations on the synaptic actions evoked in motoneurones by stronger stimulation of the sensorimotor cortex are of interest in this context A comparison of the effect on monosynaptic reflexes from extensor and flexor nerves revealed that the excitatory effects were much more marked to flexors than to extensors The curves in Fig 18 are representative Monosynaptic test reflexes of equal height were used to obtain the two curves and the initial small facilitation of the extensor test reflex is followed by inhibition This inhibitory effect could be much more marked than in Fig 18 With intracellular recording inhibitory action was found in many extensor motoneurones At cortical stimulation

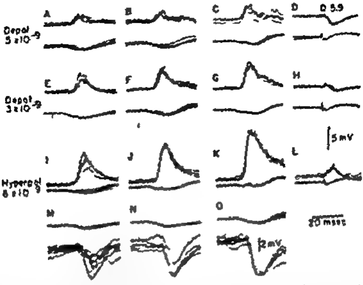

Fig 19 The intracellular records (3M KCl electrode) in A—L are from an anterior biceps-semimembranosus motoneurone and those in M—O from a gastrocnemius soleus motoneurone. The responses in A—C, E—G and I—K were evoked on stimulation of the sensorimotor cortex, the number of stimuli being shown by the shock artefact. For comparison the effect of stimulation of the quadriceps nerve at a strength of 5.9 times threshold is shown in D, H and L. In each row of records current was passed through the microelectrode with direction and strength indicated to the left. M—O were recorded from another motoneurone in the same experiment during passage of a depolarizing current of 9×10^{-9} A through the recording electrode. The lower traces in A—L and upper traces in M—O are triphasic recordings from the L_4 dorsal root entry zone.

liminal for effects on motoneurones IPSPs were evoked in 13 and EPSPs in 4 extensor motoneurones (cf. Fig. 1B and E, Fig. 13B, Fig. 15F). In flexor motoneurones on the other hand the liminal action was EPSPs in 10 cells and IPSPs in 3 (Fig. 2M, Fig. 9B, Fig. 12B and C, Fig. 14B, Fig. 16B). Also at stronger stimulation of cortex IPSPs dominated in many extensor motoneurones (Fig. 19M–O). In some motoneurones current passage through the recording electrode revealed convergence of excitatory and inhibitory action. This is illustrated by records A–L in Fig. 19. Recording was made with a KCl electrode from a hip extensor motoneurone (anterior biceps or semimembranosus) and current was passed through the electrode for reversal of IPSPs. In D, H and L are shown the IPSPs evoked by a volley in the Q nerve with the current passage indicated in each row. In D the IPSP from Q is in the hyperpolarizing direction and hence the depolarizations in A–C are EPSPs. Records E–H were obtained approximately at the equilibrium level for the IPSP from Q. During hyperpolarizing current in I–L the IPSP from Q reversed (L) and there is a large increase in the depolarizing PSPs evoked from cortex showing that the EPSP in A–C must have concealed a substantial IPSP

Stronger cortical stimulation evoked EPSPs in all but a few flexor motoneurones (Fig 12 G I) It is of interest that in 2 flexor motoneurones in which inhibition was the dominating effect of strong cortical stimulation volleys in the FRA likewise evoked inhibitory action (Fig 16B)

Discussion

The facilitatory action exerted by stimulation of the sensorimotor cortex on transmission of synaptic actions from the somatic afferents to alpha motoneurones is mediated by the pyramidal tract and can be attributed to an excitatory action on interneurones It will be reported separately that cortical stimulation evokes EPSPs in many interneurones excited by primary afferents (LUNDBERG NORRSELL and VOORHOEVE 1962) The present systematic analysis has revealed effects on *inhibitory* as well as *excitatory* paths from different classes of primary afferents to motoneurones Particularly strong effects were found on the Ia inhibitory path and on the reciprocal paths from the FRA (excitatory to flexor and inhibitory to extensor motoneurones) but effects on the excitatory and inhibitory Ib paths were also found In addition there was occasionally indication that the inhibitory path from the FRA to flexor motoneurones (*cf* ECCLES and LUNDBERG 1959a HOLMQVIST and LUNDBERG 1961) and the excitatory path from cutaneous afferents to extensor motoneurones (HAGBARTH 1952) could be facilitated The minimal latency for the facilitatory effect evoked at the interneuronal level of spinal reflex arcs by a train of cortical stimuli was 8 msec whereas the onset of facilitation of monosynaptic reflex discharges was at least 2—3 msec longer These findings are in agreement with those of LLOYD (1941) obtained with stimulation of the bulbar pyramid and are discussed in a supplementary report by LUNDBERG *et al* (1962) The cortical areas from which effects to fore and hindlimb reflexes were obtained correspond with areas from which movements can be evoked (LIVINGSTON and PHILLIPS 1957 with reference to earlier investigations)

There are many reports that stimulation of the motor cortex may evoke reciprocal actions in limb nuclei (for references see BOSMA and GELLHORN 1946 TERZUOLO and ADEY 1960) Cortical stimulation has been found to give inhibitory effects which cannot be secondary to spinal reflexes and in monkeys IPSPs evoked by cortical volleys have been recorded (PRESTON and WHITLOCK 1960 LANDGREN PHILLIPS and PORTER 1962a) The present findings raise the question if the action in motoneurones are mainly secondary to activation of interneurones of segmental reflex arcs Our finding that excitatory actions are much more prevalent in flexor than in extensor motoneurones and that inhibition is the dominating effect of cortical stimulation in many extensor motoneurones may support such a notion since excitation of flexor and inhibition of extensor nuclei are the dominating ipsilateral reflex actions In this connection it is worth noting that the few flexor cells in which inhibitory

action dominated from cortex likewise received inhibitory action from the FRA. Our results serve to elucidate SHERRINGTON's (1947) observation of a striking resemblance between the movements elicited from the motor cortex and spinal reflexes. However, the possibility that there may be interneuronal paths concerned exclusively with mediation of transmission of actions from the pyramidal tract to motoneurones cannot be excluded.

It must be emphasized that there are other mechanisms by which the pyramidal tract may influence reflex arcs. In the present experiments we did not find enhancement from cortex of the depression exerted by group I volleys from flexors on the Ia EPSP to motoneurones (FRANK and FUORTES 1957, ECCLES *et al.* 1961) but in one motoneurone of 22 tested cortical stimulation decreased the Ia EPSP. However, in further experiments with threshold measurements from Ia fibers (*cf.* WALL 1958, ECCLES *et al.* 1961, ECCLES, MAGNI and WILLIS 1962) strong cortical stimulation had no action on Ia fibres but did give a considerable increase in excitability in cutaneous afferents. Further more cortical stimulation evokes large depolarizing dorsal root potentials with spatial facilitation between effects evoked from cortex and from primary afferents (CARPENTER, LUNDBERG and NORRSELL, to be published). Reflex arcs from the FRA to motoneurones may be inhibited by this mechanism. The inhibitory effects described by LINDBLOM and OTTOSSON (1956) may have presynaptic origin.

In primates the monosynaptic connections from the pyramidal tract to motoneurone is of dominating importance in the execution of fine skilled movements (BERNHARD, BOHM and PETERSEN 1953, LANDGREN, PHILLIPS and PORTER 1962a, b). Presumably this function of the pyramidal tract in primates superimposed on the phylogenetically older actions described in the present.

It seems likely that in the regulation of motor performance in cat the control of reflex arcs from higher centres plays a considerable role. In addition to the facilitatory effects now reported, previous investigations have revealed an inhibitory control from the brain stem of these paths (except Ia). It seems purposeful that the Ia inhibitory reflex path can be mobilized from higher centres and also that the Ib reflex actions can be brought into action in a similar way (*cf.* discussion of the inhibitory control from the brain stem, ECCLES and LUNDBERG 1959b). A more complex situation prevails with the control of the reflex actions from the FRA since there is evidence of alternative pathways to motoneurones from these afferents (ECCLES and LUNDBERG 1959a, HOLMQVIST and LUNDBERG 1961, HOLMQVIST 1961). Pyramidal facilitation of interneurones may be one method used by higher centers in selection of paths from these afferents. An additional possibility is that the motor cortex utilizes the interneuronal network of the segmental reflex arcs to evoke movements of a certain pattern. It is easily imaginable that such movements would be more stereotyped than those depending on monosynaptic connexions (LANDGREN *et al.* 1962a).

Some spinal reflexes may depend on pyramidal facilitation of interneurones for their normal functioning This may be so with the reciprocal Ia inhibition from ankle flexors to ankle extensor motoneurones This action is often absent or weak in the spinal state (ECCLES and LUNDBERG 1959a) but may be considerable when facilitated from cortex (Figs 1 and 3) KUGELBERG EKLUND and GRIMBY (1960) have found that the ipsilateral nociceptive spinal reflex responses in normal humans consist of flexor and extensor reflexes one of the latter being the well known plantar flexion In cases with pyramidal disorder they found diminished or abolished extension reflexes the appearance of the Babinski sign being one of the consequences It is not unlikely that these nociceptive ipsilateral extensor reflex arcs depend on pyramidal facilitation of their interneurones The possibility that tactile placing in cat is a spinal reflex requiring pyramidal facilitation will be discussed in a forthcoming paper (LUNDBERG and NORRSELL)

Summary

The effect of stimulation of the sensorimotor cortex on synaptic actions evoked from different somatic afferent systems in alpha motoneurones has been investigated in the cat with the technique of conditioning monosynaptic reflexes and with intracellular recording from motoneurones

Stimulation of the contralateral cortex at strengths evoking small or no action in alpha motoneurones enhanced the following synaptic actions the Ia inhibitory action the reciprocal Ib actions the reciprocal actions from the FRA (group II and III muscle afferents cutaneous and high threshold joint afferents) In addition there was sometimes enhancement of the inhibitory actions from the FRA in flexor and of the excitatory actions from cutaneous afferents in extensor motoneurones

Effects on reflex actions to fore and hindlimb nuclei were evoked from the cortical areas known to influence these motor nuclei

The effects from cortex are mediated by the pyramid tract they were abolished after transection of the pyramid or the dorsal part of the lateral funicle but remained after a brain stem lesion sparing the pyramid

It is inferred that these facilitatory effects from cortex are caused by an excitatory action on interneurones of reflex arcs

Stronger cortical stimulation evoked excitatory and inhibitory actions in alpha motoneurones Excitatory actions usually dominated in flexor and inhibitory in many of the extensor motoneurones There was often evidence of mixed excitatory and inhibitory action in extensor motoneurones and inhibitory effects in flexor motoneurones were sometimes found It is suggested that these actions in alpha motoneurones are due mainly to activation of interneurones of spinal reflex arcs

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Pyramidal Effects on Lumbo-Sacral Interneurones Activated by Somatic Afferents

By

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Abstract

LUNDBERG A. U NORRSELL and P VOORHOEVE. *Pyramidal effects on lumbo-sacral interneurones activated by somatic afferents* Acta physiol scand 1962 *56* 220—229 — Intracellular recording was made from 23 interneurones in the dorsal horn and intermediate region. Stimulation of the sensorimotor cortex evoked excitatory postsynaptic potentials in interneurones receiving excitatory action from 1) group I muscle afferents 2) flexor reflex afferents, 3) cutaneous afferents In a few of the interneurones the latency was so brief as to suggest a monosynaptic connexion from the pyramidal tract. Stimulation of the sensorimotor cortex evoked inhibitory action only in one interneurone which received inhibitory action from the periphery as well The findings are discussed mainly in relation to facilitatory actions evoked by volleys in the pyramidal tract on paths from primary afferents to motoneurones

Activity in the pyramidal tract enhances excitatory and inhibitory synaptic actions evoked in motoneurones by volleys in different systems of somatic afferents (LUNDBERG and VOORHOEVE 1962) Such an action would be expected if interneurones of these spinal reflex arcs were facilitated from the pyramidal tract In the present paper it will be shown that interneurones of different classes in the lumbo-sacral cord receive excitatory action *both from* somatic afferents and from the pyramidal tract

Methods

The experimental procedure is identical with that described by LUNDBERG and VOORHOEVE (1962) In the present series the cortical stimulus strength did not exceed 1 0 mA. In two of the experiments differentiation between true interneurones and cells giving rise to ascending tracts was possible. In those experiments laminectomy

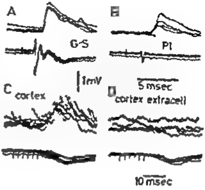

Fig 1 Intracellular recording (upper traces) from a cell in the intermediate nucleus in L7 The cell received monosynaptic excitatory action from the nerves to gastrocnemius soleus (G-S) record A and from the nerve to plantaris (Pl) in record B Volleys in all other peripheral nerves tested had no effect on this cell The effect of stimulation of the sensorimotor cortex is shown in C and in D after withdrawal of the microelectrode from the cell Differentiation between tract cells and true interneurones was not possible in this experiment The lower traces were recorded from the L7 dorsal root entry zone The records consist of superimposed traces

was made in the lower thoracic region the dorsal column was removed for a stretch of 2 cm and the cord hemisectioned The dissected conteralatral ventrolateral funicles were placed on electrodes and stimulating electrodes were also placed on the intact ipsilateral cord In this way it was possible to activate antidromically neurones with axons ascending in either spinal half The microelectrodes were usually inserted from the dorsum of the cord but the lateral approach was also used The microelectrodes were filled with 3M KCl and for intracellular recording electrodes with a resistance of about 5 MΩ proved favourable Intracellular recording was made from 23 interneurones and in addition extracellular records were taken from many interneurones When the recording was intracellular records were usually taken after withdrawal of the electrode from the cell to determine the field potentials

Results

1) *Interneurones activated by group I muscle afferents*

The records in Fig 1 were obtained from an interneurone activated by group I volleys from the nerves to gastrocnemius soleus G S (A) and from plantaris (B) but not from any other nerves The effect from cortex is shown in C and the extracellularly recorded potential in D Intracellular recording was altogether made from 8 interneurones which received monosynaptic excitatory action from group I muscle afferents and in all of them EPSPs were evoked on stimulation of sensorimotor cortex Some of the interneurones were activated by very small group I volleys, other by high threshold group I fibres and presumably these interneurones were activated by Ia and Ib fibres respectively (ECCLES ECCLES and LUNDBERG 1957) However since the group I volleys in these experiments did not separate well in fast and slow component we have abstained from grouping the interneurones in these two categories The latency in C Fig 1 is about 12 msec and the minimal latency found was 8 msec This time interval agrees well with the latency for enhancement of the reciprocal Ia inhibition which was 8—10 msec (LUNDBERG and VOORHOEVE 1962)

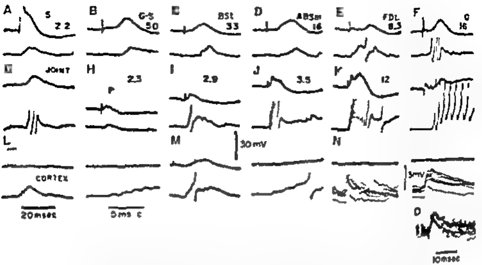

Fig 2 Intracellular recording (lower traces) from a cell receiving EPSPs from the FRA. The microelectrode was inserted in L7 from the dorsum of the cord to a depth of 1 9 mm probably to the dorsal part of the intermediary region The upper traces were recorded at the L7 dorsal root entry zone Records A—J were obtained on stimulation of the nerves indicated the abbreviations being Sur sural G S gastrocnemius-soleus PSt posterior biceps-semitendinosus ABSm anterior biceps-semimembranosus FDL, flexor digitorum and hallucis longus + the interosseus nerve Q quadriceps Joint posterior nerve to the knee joint The peroneal (P) nerve was stimulated in H K. Stimulus strengths are given in multiples of threshold strengths for the nerves. In L a short train of stimuli was given to the sensorimotor cortex at a strength just threshold for effect and in M at a somewhat higher strength but still submaximal with respect to the negative dorsal horn potential that could be evoked from cortex Record N shows the EPSP evoked by a single cortical stimulus of the same strength that was used in L. The left and right traces in record K were obtained simultaneously at two sweep speeds the slow speed to the left and the fast speed to the right below record L. A—J were taken at the slow speed indicated below L. Record O was obtained at the end of the experiment and shows the discharge evoked by a single cortical stimulus in the dissected dorsal half of the lateral funicle in L6 Differentiation between neurones giving rise to ascending tracts and true interneurones was not possible in this experiment. Records A—M consist of single traces those in N—O of many superimposed traces

2) *Interneurones activated by the FRA*

Many interneurones are activated by the flexor reflex afferents (FRA group II and III muscle afferents high threshold joint afferents and cutaneous afferents) Intracellular recording was made from 9 interneurones of this type Most of them received monosynaptic EPSP from large cutaneous afferents The records in Fig 2 are representative for an interneurone of this type The effects from high threshold muscle afferents are shown in B-F Record A shows the effect from the sural nerve G from the joint nerve and H K from the mixed peroneal nerve A train of stimuli to the sensorimotor cortex at a strength just supraliminal for evoking a negative dorsal horn potential gave an EPSP with definite waves as is best shown in the fast trace to the right in L A single cortical stimulus did produce an EPSP in this interneuron as is best shown in record N which was obtained at higher amplification The

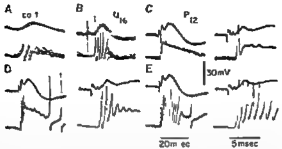

Fig 3 These intracellular records are from the same cell as those in Fig 2 The peroneal (P) nerve was stimulated at a strength of 12 times threshold in C D and E Records A—C were obtained soon after the impalement when the membrane potential was about 40 mV D was taken a few minutes afterwards when the resting potential had increased and E 15 minutes later when the resting potential was about 60 mV The left and right traces were obtained simultaneously at the two sweep speeds shown below E Observe that the channel through which the fast left traces in C—E were displayed had somewhat lower gain the calibration below C refers to the slow left trace Records A and B taken a few seconds before record C show that volleys giving smaller EPSPs than the volley in the P nerve could evoke a repetitive discharge in the cell The records in A—C consist of superimposed traces those in D and E of single traces The spike potentials in B D and E are retouched

latency for this EPSP is about 8 msec (record N) The dorsal part of the lateral funiculus was dissected afterwards in L 5—6 and mounted on recording electrodes Record O shows the mass discharge evoked by a single stimulus in the sensorimotor cortex The latency is 5 3 msec Hence it cannot be concluded with a certainty that the EPSP in L and M was monosynaptically evoked but this may very well be so since it is not necessary to assume that the fastest pyramidal fibres evoked this EPSP

The intracellular recording from the interneurone of Fig 2 was exceptionally good and some further points of interest are illustrated in Fig 3 A large longlasting EPSP was evoked from the peroneal nerve Initially record C there was only 1 spike which was followed by small waves (right record in Fig C) The records in A and B were taken immediately before that in C and show that cortical stimulation (A) and a volley in the quadriceps (Q) nerve giving smaller EPSPs than in C could evoke a train of spikes Hence it is assumed that there is a depolarization block in C. When the membral potential and spike height increased the same volley in the peroneal nerve gave the action potentials in D where in the right record there are a succession of spikes which become smaller and end with oscillatory potentials It is assumed that the decreasing size of the action potentials in D is due to incomplete invasion of the soma After further improvement of the cell the same volley in the peroneal nerve gave a longlasting train of large action potentials (record E) The records in Fig 3 are illustrated because they show that a depolarization block (GRANIT and PHILLIPS 1956) may be an artefact due to damage to the cell It is however very well possible that this cell even in C and D nevertheless discharged a train of spikes down its axon

The intracellular records in Fig 4 were also obtained from an interneurone activated from the FRA This is illustrated for the effect from DP and sural in A B and C-D respectively This interneurone received very effective ac

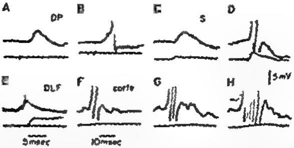

Fig. 4. Intracellular recording (upper traces) from a dorsal horn interneurone (the upper part of L7) activated by the FRA. The lower traces were obtained from the L7 dorsal root entry zone. A and B show the effect of volleys in the deep peroneal (DP) nerve obtained at threshold strength for activation of this interneurone. A group I volley in the DP nerve evoked no EPSP; the effect in A and B was obtained when the stimulus strength was raised to stimulate high threshold muscle afferents. The EPSP in A and B is probably a monosynaptic action from group II fibres but since the stimulus strength was not recorded a disynaptic action by large group II fibres cannot be excluded. The sural nerve was stimulated in C and D at strengths below and above threshold for excitation of the cell. For differentiation between tract cells and true interneurones the spinal halves (except dorsal columns) were stimulated on both sides in the lower thoracic region. The EPSP in E was evoked by a single stimulus in the intact ipsilateral dorso-lateral funicle (DLF) 10.5 cm rostral to the side of microelectrode recording. The responses in F to H were evoked on stimulation of the contralateral sensorimotor cortex with an increasing number of stimuli at constant strength. The fast sweep speed was used in A—E and the slow in F—H. The records consist of single traces.

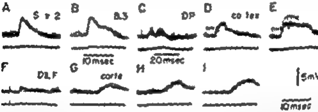

Fig. 5. Intracellular recording from a dorsal horn interneurone in the upper part of L7. Conventions and procedure as in Fig. 4. The sural nerve was stimulated in A and B at the indicated strengths given in multiples of threshold strength for the nerve. In C the DP nerve was stimulated at a strength supramaximal for group III. The sensorimotor cortex was stimulated in D, E (slow sweep speed) and in G—I (fast sweep speed). F shows the response to a single stimulus of the intact ipsilateral dorso-lateral part of the cord (DLF) 10.5 cm rostral to the site of microelectrode recording. All records consist of superimposed traces.

tivation on stimulation of the sensorimotor cortex (F–H) and a single stimulus sufficed to give a large EPSP which evoked 2 spikes (F). The latency of 6 msec is brief enough to give strong indication of monosynaptic action and on stimulation of the intact dorsolateral funicle 10.5 cm more rostrally (record E) a monosynaptic EPSP was evoked (total latency 1.7 msec). In the interneurone of Fig. 5, on the other hand, transmission from the pyramidal tract was probably disynaptic. The total latency for the EPSP evoked from cortex is almost 7 msec and the latency for the EPSP evoked on stimulation of the lateral funicle is 2.7 msec, which strongly suggests a disynaptic effect

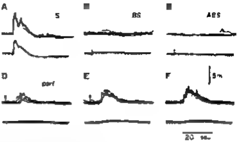

Fig 6 Intracellular recording from a dorsal horn interneurone receiving EPSPs exclusively from cutaneous afferents Procedure and conventions as in Fig 4 Stimulation of the superficial peroneal nerve in A and of the nerve to BSt (B) and ABSm (C) at strengths supramaximal for group III afferents. In D—F an increasing number of stimuli of equal strengths were given to the contralateral sensorimotor cortex The recording was made in an experiment in which differentiation between tract cells and true interneurones was possible and there was no antidromic activation of this cell from either spinal half All records consist of superimposed traces

3) *Interneurones activated exclusively by cutaneous afferents*

Some interneurones in the dorsal horn are activated exclusively from cutaneous afferents and the intracellular records in Fig 6 show that also interneurones of this type can receive excitatory action from cortex (D E) This interneurone was monosynaptically activated from the superficial peroneal nerve and it did not send its axon up the lateral funicle In the same experiment similar cells were found which gave rise to ascending axons (ECCLES ECCLES and LUNDBERG 1960 LUNDBERG and OSCARSSON 1961) It is of interest that these cells do not receive excitatory action on stimulation of the sensorimotor cortex (LUNDBERG NORRSELL and VOORHOEVE to be published)

4) *Interneurones of other types*

All interneurones cannot be classified in the above three categories (ECCLES *et al* 1960) Some represent intermixtures between them as the interneurone of Fig 7 in which EPSPs were evoked from the FRA and from group I muscle afferents of the DP nerve The excitatory action evoked by cortical stimulation is shown in A E Extracellular recording was made from several interneurones of this type and they could all be discharged from cortex Another intermixture was represented by an interneuorone monosynaptically activated by cutaneous afferents and by group I muscle afferents This interneurone received a fairly weak excitatory action from cortex

Fig 8 illustrates the only interneurone in this series receiving inhibitory action on stimulation of primary afferents and of the sensorimotor cortex.[1] This cell was monosynaptically activated from the G S nerve and from the nerves to anterior biceps and semimembranosus (ABSm) From either nerve an EPSP was evoked by a very small group I volley (A and D) which suggests that Ia fibres contributed These group I EPSPs have two phases after the fast repolarization there is a residual effect declining slowly much as has been found for cells of the dorsal spinocerebellar tract (ECCLES OSCARSSON and WILLIS 1961) A volley in the superficial peroneal (SP) nerve (record F)

[1] Added in proof In further experiments a number of interneurones of this type has been found

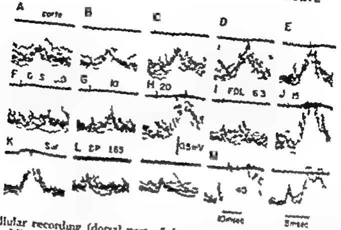

Fig 7 Intracellular recording (dorsal part of the intermediary region) from a cell receiving IPSPs from the FRA and in addition from group I afferents of the DP nerve Stimulation of the G S nerve in F—H of the FDL nerve in I and J of the sural nerve in K and of the DP nerve recorded at two sweep speeds simultaneously in L and M The strengths indicated are given in multiples of threshold strengths for the respective nerves. Stimulation of the contralateral sensorimotor cortex at constant strength with an increasing number of stimuli in A—E. All records were obtained at the slow sweep speed except the right traces in L and M Differentiation between tract cells and true interneurones was not possible in this experiment. All records consist of many superimposed traces

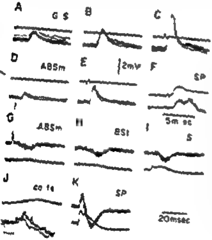

Fig 8 Intracellular recording from an interneurone of an exceptional type The microelectrode was inserted just lateral to the dorsal root entry zone to a depth of 1 3 mm Group I volleys from the G S (A C) and the ABSm (D E) nerves evoked monosynaptic EPSPs In addition a volley in the SP nerve evoked an EPSP (F) and late IPSPs were evoked by volleys in cutaneous nerves (I and K) and in muscle nerves (G and H) when the strength was raised to activate high threshold afferents On stimulation of the sensorimotor cortex (J) there was an early EPSP followed by a late IPSP of which only the beginning is seen in J Differentiation between tract cells and true interneurones was possible in this experiment and it was ascertained that this cell did not send its axon up either spinal half The records consist of superimposed traces

gave an early EPSP (probably monosynaptic) In addition late IPSPs were
evoked from all nerves as is illustrated for ABSm (G) BST (H) sural (I) and
P (K) These potentials reversed after passage of hyperpolarizing current
6 × 10⁻⁹A) through the recording microelectrode hence they were genuine
PSPs The effect of cortical stimulation is shown in J After an initial small
PSP an IPSP follows of which only the first part appears in record J

Discussion

Many investigations have been devoted to the excitatory actions exerted from primary afferents in interneurones (FRANK and FUORTES 1956 ECCLES FATT and LANDGREN 1956 KOLMODIN and SKOGLUND 1958 HUNT and KUNO 1959 ECCLES *et al* 1960) The intracellular recordings from interneurones in the dorsal horn and the intermediate region have revealed that stimulation of the sensorimotor cortex evokes EPSPs in interneurones activated by the following types of primary afferents 1) group I muscle afferents 2) FRA 3) cutaneous afferents In addition EPSPs were found in some interneurones representing intermixtures between these types In a few cases the EPSP evoked from cortex resembled the motoneuronal Ia EPSP (Fig 2) but usually the rise of the EPSP was slower indicating temporal dispersion in synaptic bombardment. The actions were evoked from the same cortical areas and at the same threshold strengths as actions proved to be mediated by the pyramidal tract (LUNDBERG and VOORHOEVE 1962) and are therefore assumed to be caused by activity in the pyramidal tract The descending excitatory actions found in many interneurones by ECCLES *et al* (1960) were probably evoked from the pyramidal tract CHAMBERS and LIU (1957) have shown that in cat the pyramidal tract has a preterminal distribution to the dorsal horn and the intermediate nucleus WALL MC CULLOCH, LETTVIN and PITTS (1955) using a *new electrophysiological approach found terminal arborization of the pyr*amidal tract in the ventro-lateral part of the dorsal horn

The present results support the hypothesis that the facilitatory effect from the pyramidal tract on paths from primary afferents to alpha motoneurones is due to excitatory action on interneurones of these paths (LLOYD 1941 LUNDBERG and VOORHOEVE 1962) However the destinations of axons from the interneurones recorded from are not known and volleys in the pyramidal tract are known also to facilitate transmission from primary afferents to ascending spinal pathways (MAGNI and OSCARSSON 1961 LUNDBERG NORRSELL and VOORHOEVE to be published) and to primary afferents (CARPENTER LUNDBERG and NORRSELL to be published)

In interneurones monosynaptically activated from group I muscle afferents the latency for the EPSP evoked from cortex ranged from 8—13 msec There is good agreement between these values and the latency for the onset of facilitation from cortex of Ia inhibition in montoneurones (about 8—10 msec Fig 3 LUNDBERG and VOORHOEVE 1962) A volley from cortex takes about 5 msec to reach the lumbo-sacral cord (Fig 2) and it can therefore not be assumed that the pyramidal tract has monosynaptic connections with Ia inhibitory interneurones There was on the other hand evidence for monosynaptic EPSPs from cortex in some interneurones activated from the FRA It *is not* possible to exclude that such interneurones may belong to paths to motoneurones although the latency for facilitation from cortex of actions from

cutaneous afferents to motoneurones is about 10 msec (LLOYD 1941, LUNDBERG and VOORHOEVE 1962), because in the majority of the interneurones the EPSP from cortex has a slow rising phase and repetitive stimulation is usually necessary to detect an action on the effect of conditioning volleys on monosynaptic test reflexes. LLOYD (1941) found that small nuclear elements in close proximity with the tract constitute the immediate relay and that the assumed premotoneurone relay in the intermediate nucleus has a nuclear delay of several milliseconds. A nuclear delay of this order can be accounted for by the slow rise of the EPSP now found in the interneurones of this region. The time course of the EPSPs in interneurones is also of interest when considering the relationship between effects from the pyramidal tract on motoneurones and on paths en route to motoneurones. The latency for the latter actions is several msec shorter than the latency for the action evoked in the motoneurones (LLOYD 1941, LUNDBERG and VOORHOEVE 1962, Fig. 3 and 16). Because of the time course of EPSPs evoked from cortex this long latency is compatible with the suggestion by LUNDBERG and VOORHOEVE (1962) that motoneuronal actions evoked from cortex may be secondary to activation of interneurones of spinal reflex arcs.

LLOYD (1941) found that pyramidal volleys could inhibit the resting discharge in some interneurones and pointed out that reciprocal innervation of motoneurones could be exerted this way. Activation of excitatory and inhibitory interneurones of spinal reflex arcs now provides a more plausible explanation for such reciprocal actions from the pyramidal tract. Before ascribing inhibitory action from any source to interneurones differentiation between true interneurones and tract cells is required. Ventral spino-cerebellar tract cells in particular receive strong inhibitory actions both from the periphery and from the pyramidal tract (OSCARSSON 1957, ECCLES, HUBBARD and OSCARSSON 1961, MAGNI and OSCARSSON 1961). In the present investigation cortical stimulation gave rise to an IPSP in one interneurone, but an IPSP was also evoked in this cell by volleys in primary afferents. Corresponding to what has been suggested for motoneurones and for VSCT cells it is possible that also in this case there was a causal relationship between these two effects, i. e. that there may be convergence from the periphery and from cortex of excitatory action to inhibitory interneurones acting on interneurones.

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Estrogenic Activity of Some Isoflavone Derivatives

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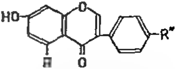
Abstract

NILSSON, A. *Estrogenic activity of some isoflavone derivatives.* Acta physiol. scand. 1962. 56. 230—236. — Five isoflavones biochanin A, genistein, formononetin, daidzein and 5,7-dihydroxyisoflavone have been synthesized. Their estrogenic activity was assayed by the mouse uterine weight method under different administration routes. Daidzein, in contrast to the four other isoflavones, was not absorbed when injected subcutaneously and its estrogenic activity was in consequence of this negligible by subcutaneous injection. By oral or intraperitoneal administration the estrogenic activity of daidzein and genistein was approximately 10^{-5} times that of diethylstilbestrol. Biochanin A and 5,7-dihydroxyisoflavone were about half as active estrogens as daidzein and genistein. Formononetin was inactive. A free para hydroxy group in the phenyl nucleus of the molecule is present in daidzein and genistein but is lacking in biochanin A, formononetin and 5,7-dihydroxyisoflavone. The presence of a free para hydroxy groups therefore seems to be of importance for the estrogenic activity.

The isoflavones genistein (POPE and WRIGHT 1953) and biochanin A (POPE *et al.* 1953) account for a considerable part of the estrogenic activity of red clover. A third isoflavone compound, formononetin, with negligible estrogenic activity has also been found in red clover (BATE-SMITH, SWAIN and POPE 1953). The three compounds mentioned above have earlier been isolated from and identified in plants from widely different species. Soybean oil meal which is an important feed supplement is rich in the glucosides of genistein and daidzein (WALTZ 1931). The structural formulas of the isoflavones are shown in Fig. 1.

Fig 1 Structural formulas of the isoflavones

Genistein	R = OH	R = OH
Biochanin A	R = OH	R = OCH
Daidzein	R = H	R = OH
Formononetin	R = H	R = OCH
5-7 OH isoflavone	R = OH	R = H



The estrogenic activity of the isoflavones is very low only 10^{-5} times that of diethylstilbestrol or estradiol Large amounts of isoflavone rich forage are however consumed by farm animals Sufficient estrogenic substances may there fore be ingested to exert an important influence on the endocrine state in the animals

Biochanin A was found to be demethylated to genistein in rumen fluid by the influence of micro-organisms (NILSSON 1961 b) and in liver by enzyme systems in the microsomes (NILSSON 1961 c) (NILSSON to be published) Formononetin is also demethylated by micro-organisms in the rumen and by liver enzymes and is thereby transformed into daidzein (NILSSON to be published) Formononetin is considered to be an inactive (BRADBURY and WHITE 1953) or weakly estrogenic compound (CHENG *et al* 1954) As this isoflavone occurs richly in red clover (VIRTANEN and HIETALA 1958) it may nevertheless contribute to the total estrogenic influence from the food when it has been converted into the more active daidzein in the rumen and the liver There are however diverging reports in the literature about the estrogenic activity of daidzein BRADBURY and WHITE (1953) found it quite unable to increase the uterine weight in immature mice when these were injected with an oil solu tion of the compound According to CHENG *et al* (1954) daidzein appeared to be the most active estrogenic isoflavone with the uterine weight response tech nique They fed the compound mixed with the basal ration to immature mice in a preliminary experiment with 5 animals

The primary aim of this work was to re investigate the estrogenic activity of the four naturally occurring isoflavones mentioned above Besides the effect on the estrogenic activity of different administration routes was studied

In the hope of obtaining some further informations on the hydroxyl arrange ment necessary for the biological activity of the isoflavones 5 7 dihydroxy isoflavone was also examined This compound is lacking the 4 hydroxy group which is present in the four other isoflavones mentioned above

Experimental

Material and methods

Biochanin A genistein formononetin daidzein and 5-7-dihydroxy isoflavone were synthesized by modifications (NILSSON GRONOWITZ and EKMAN 1961) of known methods (BAKER *et al* 1953 a

The estrogenic activity was estimated by the uterine weight method The specific ac tivity of biochanin A has earlier been shown to be approximately the same within the

Table I. The uterine weight response in mice given daily 2 g of a test ration containing 5 mg of the isoflavones

Treatment	No. of mice	Weight in mg of uteri — Freshly prepared: Mean	S.D.	Estimated potency in terms of diethylstilbestrol	Weight in mg of uteri — Fixed in Bouin's fluid: Mean	S.D.	Estimated potency in terms of diethylstilbestrol
Test period I							
Biochanin A	16	21.4	5.1	$0.56 \cdot 10^{-4}$	16.8	3.9	$0.57 \cdot 10^{-4}$
Genistein	16	34.0	5.5	$0.80 \cdot 10^{-4}$	27.3	4.2	$0.80 \cdot 10^{-4}$
Formononetin	15	9.6	1.1	0	7.4	0.9	0
5-7-dihydroxy isoflavone	16	14.9	4.7	$0.32 \cdot 10$	12.6	4.0	$0.40 \cdot 10^{-4}$
α-diethylstilbestrol							
none	15	10.6	3.0		7.9	2.2	
0.02	16	16.4	2.2		12.2	2.0	
0.04	16	36.02	5.4		27.7	4.2	
0.05	6	41.6	7.5		37.2	6.4	
Test period II							
Daidzein	16	30.4	7.9	$0.92 \cdot 10^{-4}$	24.1	6.2	$0.97 \cdot 10^{-4}$
α-diethylstilbestrol							
0.01	16	8.5	1.5		7.0	1.1	
0.04	15	26.5	6.7		20.9	4.8	

dose range of 1.5—5.0 mg per day by oral administration (NILSSON 1961 a). An assumption has been made that this relation is valid also for the other four isoflavones examined in the present study.

The tests were performed as "spot tests" with one group of the isoflavone and two groups of diethylstilbestrol and 0-control in each experiment. The isoflavones and the standards were administered by the same route, injection or feeding. The estrogenic activity of the isoflavones was estimated in terms of diethylstilbestrol by comparison of the uterine weight response to a given amount of isoflavones with a dose response line of the diethylstilbestrol standards. Differences in handling of the preparation and weighing of the uteri during different assay periods were hereby eliminated. The estimations of estrogenic activity agreed tolerably well if they were made from curves based on the weights of either freshly prepared or fixed uteri.

In the injection experiments peanut oil or propylene glycol were used as a solvent. Supersaturated solutions of the isoflavones in these solvents were prepared by heating and slowly cooling of the solutions to body temperature before injection. The largest dose which could be conveniently given did not exceed 1.25 mg per 0.05 ml of solution. Injections were given subcutaneously or intraperitoneally twice a day for 3 days. The animals were killed 24 hours after the last injection.

In the feeding experiments the isoflavones were incorporated into the basal diet. The compounds were dissolved in acetone, then mixed with the ration and the solvent re-

Table II The uterine weight response in mice given subcutaneous injections of the isoflavones

Treatment	Daily dosage	Solvent	No of mice	Weight in mg of uteri, Freshly prepared: Mean	S D	Estimated potency in terms of diethylstilbestrol	Weight in mg of uteri, Fixed in Bouin's fluid: Mean	S D	Estimated potency in terms of diethylstilbestrol
Test period I									
Genistein	2.5 mg	propylene glycol	15	37.0	3.6	$1.20\ 10^{-}$	24.9	2.1	$1.10\ 10^{-4}$
Daidzein	2.5 mg	propylene glycol	15	8.5	1.6	$0.10\ 10^{-}$	6.6	1.2	$0.12\ 10^{-4}$
Daidzein	1.25 mg	peanut oil	15	7.2	1.2		5.3	0.9	[b]
Diethylstilbestrol	*none*	propylene glycol	14	6.8	1.4		5.1	1.4	
	0.01 γ	propylene glycol	15	13.8	2.4		10.2	2.0	
	0.02 γ	propylene glycol	14	25.6	4.9		18.9	3.7	
Test period II									
5-7-dihydroxy isoflavone	1.25 mg	propylene glycol	17	8.6	2.0	$0.35\ 10^{-}$	5.9	1.3	$0.40\ 10^{-}$
Diethylstilbestrol	*none*	propylene glycol	14	6.8	1.4		4.4	1.1	
	0.01 γ	propylene glycol	15	11.4	2.4		7.3	1.5	
	0.015 γ	propylene glycol	15	14.3	2.3		9.0	1.7	

[a] The differences in uterine weight between mice given 0 control injections and those given isoflavone injections are statistically significant ($P < 0.05$)

[b] References are lacking of animals injected with standard doses of diethylstilbestrol in peanut oil

Table III The uterine weight response in mice given intraperitoneal injections of the isoflavones

Treatment	Daily dosage	Solvent	No of mice	Weight in mg of uteri		Estimated potency in terms of diethylstilbestrol	Weight in mg of uteri		Estimated potency in terms of diethylstilbestrol
				Freshly prepared			Fixed in Bouin's fluid		
				Mean	S D		Mean	S D	
Genistein	1.25	propylene glycol	19	11.4	1.7	$0.72 \cdot 10^{-4}$	7.8	1.1	$0.71 \cdot 10^{-4}$
Daidzein	1.25	propylene glycol	19	13.5	2.2	$0.85 \cdot 10^{-4}$	9.5	1.6	$1.04 \cdot 10^{-4}$
Daidzein	1.25	peanut oil	10	11.2	2.1	[1]	7.3	1.4	[1]
Diethylstilbestrol	none	propylene glycol	11	6.5	1.0		5.2	0.9	
	0.01 γ		1?	12.6	1.5		8.3	1.0	
	0.015 γ		1?	14.5	1.7		10.1	1.5	

[1] References are lacking of animals injected with standard doses of diethylstilbestrol in peanut oil.

moved Water was added to the dry powdered ration and the resulting dough was formed into small cubes and dried This process prevents food losses as it is more difficult for the mice to waste the cubes compared with the powdered food Each mouse consumed an average of 2 g of food daily and this ration was fed to the mice for 4 to 5 days The daily intake was controlled and the animals were killed when they had left no food remains during at least 3 days

Female mice three weeks old and weighing 8—10 g were used for the tests The mice were randomly bred and of the strain Navy Medical Research Institute (furnished by Anticimex Norrviken) The uteri were removed trimmed of adhering tissues blotted between filter papers and weighed The uteri were also as a weight control fixed in Bouin's fluid for 24 hours gently dried and re weighed

Results and discussion

The results from feeding experiments with the 5 isoflavones during two test periods are recorded in Table I The experiment with daidzein was performed some months later (Test period II Table I) than the experiment with the other isoflavones (Test period I Table I)

Biochanin A genistein and daidzein are all active estrogens with an activity 10^{-4} times that of diethylstilbestrol Formononetin is inactive as an estrogen The numerical values of the estrogenic activity of biochanin A and genistein were a little lower than in earlier experiments of the present author

Fig 2 The uterine response to subcutaneous inject on of da d ein (left p cture) and genistein (right picture)

(NILSSON 1961 a) A plausible explanation for this finding is difficult to offer but differences in the mouse material might be one reason for the different results During the earlier experiments (NILSSON 1961 a) another strain of mice were used than in the present one

Genistein and daidzein were also administered by subcutaneous injection (Test period I Table II) and by intraperitoneal injection (Table III) When given subcutaneously genistein was still active while daidzein had almost no effect (Fig 2) The difference in action of daidzein depending upon mode of administration is probably due to its low solubility resulting in development of crystals in the skin after subcutaneous injection which have been observed in the present study

The structural requirements which are necessary for estrogenic activity of isoflavones are difficult to postulate BRADBURY and WHITE (1953) regarded a 5-hydroxy group as essential for estrogenic activity The results of CHENG *et al* 1954 and of the present investigation show that daidzein lacking a 5 hydroxy group is as active an estrogen as genistein

Hydroxyl groups in para position in the phenyl nucleus of the isoflavones seems to be of importance for the estrogenic activity 5 7 hydroxy isoflavone is said to have about one fourth of the activity of genistein (BAKER, HARBONE and OLLIS 1953 b) 5 7-dihydroxy isoflavone lacking a hydroxyl group in the phenyl nucleus has in the experiments reported here less than half of the

estrogenic activity of genistein The compound gave the same estimated potency in terms of diethylstilbestrol when it was given orally (Test period I Table I) as when it was subcutaneously injected (Test period II, Table II)

The uncertainty in all determinations of true estrogenic activity must be stressed No absolute significance can be attached to the observed potency of a substance Many factors, besides the original chemical structure influence the estrogenic activity of a compound For instance mode of administration sensitivity of the animals used rate of absorption and destruction and metabolic modifications of the compounds like the above mentioned demethylation (NILSSON 1961 c), hydroxylation (WILLIAMS 1959, p 546) and other unknown metabolic steps can alter the estrogenic activity

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Acta physiol scand 1962 56 237—243

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The Removal of Dietary Chylomicrons and Artificial Fat Emulsions from the Circulation of Rats

By

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Received 15 March 1962

Abstract

Edgren B and H C Meng *The removal of dietary chylomicrons and artificial fat emulsions from the circulation of rats* Acta physiol scand 1962 *56* 237—243 — The purpose with the investigation was to compare the removal of three different artificial intravenous fat emulsions from the blood of nonfasted rats with the removal from blood of washed chylomicrons collected from rats fed cottonseed oil The injected dose of glycerides varied between 300 and 1 000 mg/kg body weight The elimination pattern of the injected particulate fat was determined turbidimetrically : The chylomicrons as well as the different emulsions showed an exponential type of disappearance during the major part of the elimination process Two of the tested emulsions contained vegetable oil stabilized with phospholipids These emulsions showed an elimination rate very similar to that of chylomicrons The third emulsion (SR) lacking phospholipids and containing vegetable oil stabilized with synthetic agents disappeared from blood significantly faster than chylomicrons With all the injected substrates the elimination rate decreased with increasing fat loads

The removal from the circulation of injected chylomicrons has been studied in rats by among others French and Morris (1957) Borgstrom and Jordan (1959) and Olivecrona George and Borgstrom (1961) The problem has been studied in dogs by McCandless and Zilversmit (1958) The disappearance from the circulation of fat emulsions has been investigated in rats by Waddell *et al* (1953) and in dogs by Edgren (1960 a) Most of the mentioned authors have stressed the importance of the injected dose of fat the

Table I Composition of used fat emulsions

	Phosphatidic cottonseed oil emulsion		Non phosphatide cottonseed oil emulsion		Soya oil emulsion	
Glyceride component	15	cottonseed oil	15	cottonseed oil	15	soya oil
Phospholipid component	1 2	soybean phosphatides		—	1.2	egg phosphatides
Other emulsifiers	0 3	Pluronic F-68 (polyoxyethylene polypropylene glycol)	0 3 1 25 0.5	Pluronic F-68 polyethylene glycol 400 stearate TEM 4T		—
Water phase	5	glucose	5	glucose	2 5	glycerol

[1] TEM 4 T = acetylated tartaric acid ester of monoglycerides.

slope of the exponential elimination curve decreasing with increased doses Few workers have compared in identical experiments dietary chylomicrons with different fat emulsions however The present study compares in the rat the disappearance of chylomicrons with three artificial intravenous fat emulsions

Experimental

Nonfasted Sprague Dawley rats weighing over 300 g were fed 3 ml cottonseed oil by stomach tube Within one hour these rats were anesthetized with ether and a thoracic duct fistula made according to the method of BOLLMAN CAIN and GRINDLAY (1948) Lymph was collected over night on ice bath concentrated and washed with saline at 30 000 × G in a superspeed centrifuge The triglyceride content of the concentrated chylomicron suspension was determined according to the method of CARLSON (1959) It was tried not to use lymph older than three days

Three fat emulsions were used for comparison with chylomicrons Cottonseed oil emulsion[1] stabilized with soybean phosphatides cottonseed oil emulsion[2] lacking phospholipids and soya oil emulsion[3] stabilized with egg phosphatides The composition of the emulsions is shown in Table I

For the elimination experiments nonfasted Sprague Dawley rats of both sexes weighing 120—200 g were used The injections were made in the tail vein the injection time seldom exceeding 15 sec Chylomicrons were administered in doses ranging from 230 to 1 000 mg/kg and the doses of cottonseed oil soybean phosphatide emulsion and soya oil emulsion ranged from 300 to 1 000 mg/kg The non phosphatide cottonseed oil emulsion disappeared so rapidly at doses lower than 500 mg/kg that elimination curves were very difficult to calculate

[1] "Infonutrol s... AB Astra S...ilje Sweden
"SR -emulsion ... the Southe... ...ion Research and Development Division
U S Departm... ...lture New ... U S A
Intralipid ... Vitrum ... Sweden

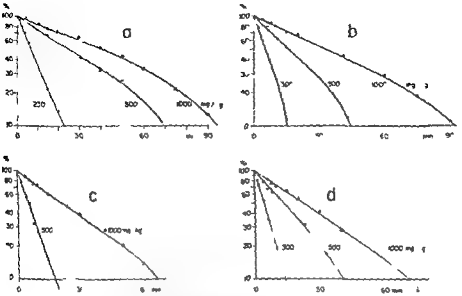

Fig 1 Removal from rat blood of chylomicrons and three different fat emulsions a) chylomicrons b) cottonseed oil-soybean phosphatide emulsion c) "SR" -emulsion a non phosphatide cottonseed oil emulsion d) soya oil-egg phosphatide emulsion

These rats were anesthetized with Nembutal sodium intraperitoneally (5 mg/100 g body weight) Arterial blood samples were taken through a polyethylene tubing in one carotid artery The blood was allowed to run freely on a paraffin block and 0 1—0 2 ml was taken for determination of particulate fat in whole blood according to the method of GEYER MANN and STARE (1948)

In each experiment the time necessary for the removal of both 50 and 90 per cent of the injected dose was calculated

Results

Typical elimination curves plotted semilogarithmically, with chylomicrons and the different artificial fat emulsions are shown in Fig 1 The similarity in characteristics for the curves obtained with the different substrates is obvious The elimination of the smaller doses usually seemed to follow a single exponential regression Doses of 500 mg kg and more however were in general eliminated exponentially during the removal of 60—80 per cent of the injected dose After this the curves usually deviated from the exponential curve downward indicating a slight increase in the rate of removal

In Fig 2 and 3 is compared the elimination of different doses of chylomicrons and emulsions At 500 mg/kg sufficient material for statistical calculations was available In Table II are given the average half time for chylomicrons and emulsions with this dose of triglycerides At this dose the difference between the half time for chylomicrons and the phosphatidic cottonseed oil emulsion

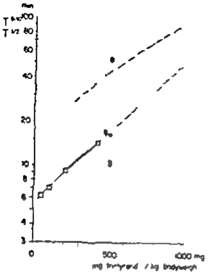

Fig 2 Relation between injected dose of triglycerides and chylomicron removal rate Open circles rate of removal expressed as time required for disappearance from the circulation of 90 % of the injected dose Solid circles half time for the injected dose Squares half time for chylomicrons reported by FRENCH and MORRIS (1957)

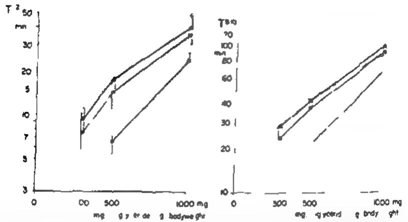

Fig 3 Relation between dose and removal rate of three different artificial fat emulsions Left part of fig Relation dose half life Right part of fig Relation between dose and time for 90 % removal of the injected dose ▲——▲ Cottonseed oil emulsion with phosphatides ——— Soya oil emulsion with phosphatides ■——■ Non phosphatide SR -emulsion The dotted lines show the dose-elimination rate relation with chylomicrons showed more in detail in fig 2

was not significant The soya oil emulsion was removed somewhat but not significantly faster than chylomicrons The non phosphatide cottonseed oil emulsion SR was removed significantly faster than chylomicrons however

At 300 500 and 1 000 mg triglyceride/kg the phosphatidic cottonseed oil emulsion was removed at a slightly slower rate than the soya oil emulsion The difference was not statistically significant, however

Table II Half time in rat blood of dietary chylomicrons and three types of artificial fat emulsions The injected dose was 500 mg triglyceride/kg body weight Each figure represents the mean value of the half time and its mean error Six animals in each group

Chylomicrons (min)	Non phosphatidic cottonseed oil emulsion (min)	Phosphatidic cottonseed oil emulsion (min)	Soya oil emulsion (min)
16.2 ± 2 5	6 6 ± 1.2	17 5 ± 2 4	14.2 ± 3 1

SR emulsion was at both 500 and 1 000 mg/kg removed much more rapidly than both chylomicrons and the other two emulsions The differences between SR emulsion and the other injected fats was statistically significant

The rate of removal as measured by T1/2 decreased with increased dose of chylomicrons and emulsions

Discussion

Nonfasted rats were used since it has been shown by ASHERMAN *et al* (1961) that nonfasted rats eliminate emulsions from their blood as rapidly as fasted rats

From the presented data it seems evident that if any comparisons between the removal from the circulation of chylomicrons and emulsions are to be made identically large doses should be compared since the rate of removal changes so rapidly with changes in dose Thus for instance if the disappearance of 230 mg kg of chylomicrons is compared with that of 500 mg/kg triglyceride as soya oil emulsion chylomicrons seem to be eliminated much more rapidly which is not the case

In general the tested emulsions were removed from blood at a similar or more rapid rate than chylomicrons There were however differences between the emulsions Thus no general statement can be made on the removal of fat emulsion from the blood one brand of emulsion tells very little about other types of preparations

The phosphatidic cottonseed oil emulsion was the emulsion that was most similar to chylomicrons Soya oil emulsion was somewhat faster This might possibly be explained by the fact that the chylomicrons were collected after feeding of the rats with cottonseed oil the same oil as that in the phosphatidic cottonseed oil emulsion Soya oil contains more unsaturated fatty acids than cottonseed oil and might therefore be metabolized somewhat more rapidly It would therefore be of interest to investigate the removal from blood of chylomicrons collected after feeding of fatty acids of different chain length and degree of saturation.

The non phosphatide cottonseed oil emulsion SR was removed from blood significantly faster than the chylomicrons and the other two emulsions Thus

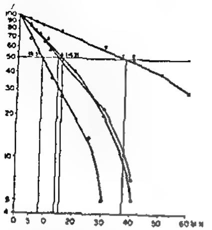

Fig 4 Disapp arance of three artificial fat emulsions and washed dietary chylomicra from the blood circulation of dogs Dose 500 mg triglycerides kg chylomicra non pho phatide emulsion (SR 659) cottonseed oil emulsion, stabilized with phosphatides (Infonutrol) △ soya oil emulsion stabilized with egg phosphatides (Intralipid) Figures in parentheses are T 1/2 in minutes

the emulsifying system is of great importance, since one of the other emulsions contains the same oil The very rapid disappearance from the blood of non phosphatide cottonseed oil emulsion is in line with dog experiments with a non phosphatide cottonseed oil emulsion by EDGREN (1960 b) Emulsions lacking phospholipids are in general more unstable than preparations where phospholipids serve as stabilizers Instability in the blood might cause embolization of particles in the blood capillaries This might explain the rapid disappearance of such emulsions from the circulating blood

The experiments confirmed earlier observations that the removal rate of both chylomicrons (FRENCH and MORRIS 1957) and fat emulsions (EDGREN 1960 a) decreased with increased dosage of fat

The turbidimetric determination of fat by GLYER, MANN and STARE (1948) res only the amount of light scattering fat particles This explains in part why the two phase elimination curve for chylomicrons that has been shown by FRENCH and MORRIS (1957) with C^{14} labeled chylomicrons was not reproduced in our experiments since the second and more slow exponential part of the elimination curve with chylomicrons is probably caused by the slower removal of chylomicron phospholipid from the circulation (OLIVECRONA GEORGE and BORGSTROM 1961) In spite of this there was a good agreement between our observed half times with chylomicrons and the results of FRENCH and MORRIS (1957) as seen in Fig 2

The presented experiments in rats indicate that out of three tested commercial emulsions two showed a removal rate that did not differ significantly from that of chylomicrons One of the emulsions disappeared from the circulation faster than all the other substrates however One should not conclude that an emulsion that is removed fast from the circulation is a more useful preparation for nutrition purposes than an emulsion with a slower rate of removal In that case one might come to the conclusion that some fat emulsions are utilized better than dietary chylomicrons, which is probably not true

Several workers have found that artificial emulsions are taken up by reticuloendothelial elements in the spleen liver and the lungs more than dietary fat does (MURRAY and FREEMAN 1951) This accumulation in the RES might explain why some emulsions are eliminated from the blood stream more rapidly than chylomicrons

Fig 4 shows preliminary results from similar experiments in non anesthetized dogs Such experiments performed in analogy with our rat experiments indicate that in dogs phosphatide stabilized emulsions when compared with homologous chylomicrons are removed more slowly from blood in contrast to what was observed in the rat experiments Non phosphatide SR emulsion however was also in dogs eliminated faster than chyle Our preliminary results in dogs confirming the observations in dogs by BERRY and IVY (1948) are mentioned just to show that there might exist variations between species

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Acta physiol scand 1962 56 244—248

From the Institute of Occupational Health Helsinki Finland

Distribution of Ingested Palmitic Acid-1-C^{14} between Rat Serum Lipoprotein Fractions

By

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Abstract

Karvinen E, O Koskimies and M Miettinen *Distribution of ingested palmitic acid 1 C^{14} between rat serum lipoprotein fractions* Acta physiol scand 1962 *56* 244—248 — Uptake of palmitic acid 1 C^{14} in albumin plus α_1 lipoprotein fraction in α_2 lipoprotein β lipoprotein and chylomicron fractions separated by paper electrophoresis was followed from 3 to 76 hours after oral administration of palmitic acid 1 C^{14} Except the initial high activity in the chylomicrons the highest activity was found in the albumin plus α_1 lipoprotein fraction Apparent turnover time of this fraction between 3 and 18 hours after ingestion of the label was about 12 hours Activity of the α_2 lipoprotein fraction was not much altered up to 28 hours Activity of the β lipoprotein fraction seemed to follow the activity of the chylomicron fraction to a greater extent

After its intestinal absorption 70—90 % of the palmitic acid absorbed can be recovered from the lymph draining the small intestine (Bloom *et al* 1950) It has been shown that long chain fatty acids when fed in the free form appear in the chyle mainly as triglycerides (Borgström 1951 Reiser and Bryson 1951) contained in the chylomicrons After the chylomicrons enter the circulation from the thoracic duct the liver presumably takes up the main part of the chylomicron triglycerides (Borgström and Jordan 1959) In the liver the triglycerides are rapidly hydrolyzed and their fatty acids given off to the circulation probably as free fatty acids (Olivecrona George and Borgström 1961) Plasma non esterified fatty acids are mainly carried by the albumin

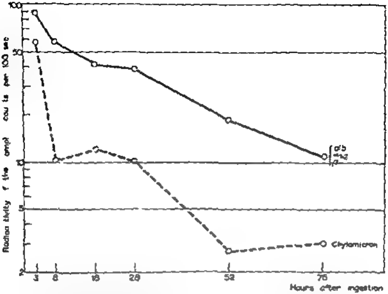

Fig 1 Semilogarithmic plot of radioactivity of the chylomicron fraction and the sum of the radioactivities found in the lipid of all lipoprotein and albumin fractions after ingestion of palmitic acid 1-C^{14} Data from 9 to 6 rats.

fraction (Kendall 1941 Goodman 1958) and only 1—2 % of the blood non esterified fatty acids are bound to red cells and low and high-density lipoproteins (Goodman)

In the present study distribution and turnover of ingested palmitic acid 1 C^{14} in the different lipoproteins of the rat serum were investigated

Material and Methods

Female rats of Wistar strain weighing 200—250 g were caged individually and given a stock diet for 3 weeks ad libitum Then the rats were fasted overnight and fed at 11 a m 4 μc (0 5 mg) of palmitic acid 1 C^{14} in 2 g of entire wheat flour The labelled palmitic acid was supplied by the Radiochemical Centre Amersham, England Palmitic acid 1 C^{14} was dissolved in ether and poured on the dry entire wheat flour Then the dry diet was made to a paste by adding water The rats ate the paste in about 15 min

From 9 rats blood samples of about 1 ml were taken under ether anesthesia 3 8 18 and 28 hours after the ingestion of the labelled palmitic acid From 6 rats the blood samples were taken 28 52 and 76 hours after the ingestion of the labelled palmitic acid

Fractionation of the serum lipoproteins was carried out according to the paper electrophoretic method of Nikkila (1953) as modified and described elsewhere (Miettinen 1957)

The electrophoresis paper was cut into stripes containing albumin plus α_1 globulin plus α_1 lipoprotein α_2 globulin plus α_2 lipoprotein β globulin plus β lipoprotein and the area around the starting line containing chylomicrons The stripes were boiled in

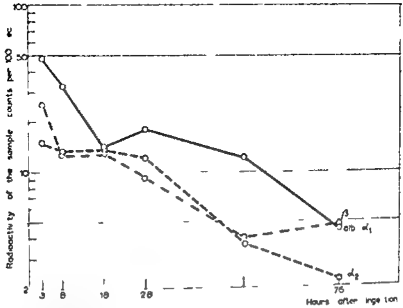

Fig 2 Radioactivities found in the lipid of albumin plus α lipoprotein fraction of α_1 lipoprotein and β lipoprotein after ingestion of palmitic acid 1 C[14] Data from 9 to 6 rats

10 % ethanolic KOH for 2 hours The mixture was then acidified by adding H_2SO_4 and the acidified mixture was allowed to stand overnight Then the lipids were taken up in petroleum ether and washed with 50 % ethanol and water The washed petroleum ether was transferred to steel planchets and assayed for radioactivity using a thin mica window Geiger tube (20th Century EW 3 H) and an EKCO Automatic Scaler N 530 F

Results

In Fig 1 the radioactivity found in the electrophoretically separated chylomicron fraction and the sum of the radioactivities found in all the lipoprotein and albumin fractions are shown as a function of time

The radioactivity in the chylomicron fraction diminished very rapidly from 3 to 8 hours after the ingestion of palmitic acid 1 C^{14} On the other hand the total activity in the lipoprotein and albumin fraction seemed to decline rather linearly The apparent turnover time of this fraction was estimated to be approximately 32 hours

In Fig 2 the radioactivities found in the lipid of the albumin plus α_1 lipoprotein fraction The activity seemed to decline linearly up to 18 hours after the ingestion of palmitic acid 1 C^{14} Thereupon the decline ceased for one day The turnover time of this fraction during the initial linear phase of decay between 3 and 18 hours was estimated as 12 hours

In Fig 2 the activity found in α_2 lipoprotein is shown The activity seems to be steady until 28 hours after the ingestion of the label Then a slow turn

over is noted having a turnover time of the same order of magnitude as that shown in Fig 1 for the sum of all the lipoprotein and albumin fractions

In Fig 2 the initial activity found in β lipoprotein is smaller than in the chylomicron fraction and the initial decline is not so rapid as in the chylomicron fraction From 8 hours after ingestion of palmitic acid 1 C^{14} the changes of radioactivity in the β lipoprotein and chylomicron fractions are very similar

Discussion

After injecting rats with lymph obtained from rats fed glycerides containing trace amounts of palmitic acid 1 C^{14} (OLIVECRONA *et al* 1961) it was found that the glyceride activity in the plasma disappeared rapidly having a half life of 7 min the phospholipid disappeared with a half life of half an hour and non esterified fatty acid was increased rapidly in the plasma reaching a peak in 15 min and then disappeared rapidly The half life for injected albumin bound non esterified palmitic acid 1 C^{14} was found to be about 1 minute in the rat (OLIVECRONA *et al* 1961) Since about 98 % of plasma non esterified fatty acids are carried by the albumin fraction (GOODMAN 1958) it would follow that the disappearance of plasma non-esterified palmitic acid would run parallel with the entry of non esterified palmitic acid in the plasma with a delay of approximately one minute From the data of OLIVECRONA *et al* (1961) on the disappearance of labelled chylomicrons it may be estimated that the non esterified palmitic acid 1 C^{14} enters the plasma about 15 min after the introduction in the circulation of palmitic acid 1 C^{14} labelled chylomicrons Thus it can be calculated that the delay in the radioactivity of the albumin bound non-esterified palmitic acid 1 C^{14} would be 15 + 1 minute after the appearance of the palmitic acid 1 C^{14} labelled chylomicrons in the blood plasma Therefore if the palmitic acid 1 C^{14} given in the present study would have been in the serum in the non esterified form then its disappearance from the serum would have followed the activity curve of the chylomicrons (Fig 1) with a delay of 15 + 1 = 16 min only Since the decline in the activity of the albumin + α_1 lipoprotein fraction is not so rapid as the initial decline in the activity of the chylomicron fraction it is probable that the activity of the albumin + α_1 lipoprotein fraction is not predominantly bound to the albumin in the form of non esterified fatty acid but is mainly contained in the α_1 lipoprotein

Supported by a grant from S gr d Jusel us Foundation

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Acta physiol scand. 1962 56 249—257

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The Accumulation and Metabolism of C^{14}-labelled Nicotine in the Brain of Mice and Cats

By

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Abstract

APPELGREN L. E E HANSSON and C G SCHMITERLÖW *The accumulation and metabolism of C^{14}-labelled nicotine in the brain of mice and cats* Acta physiol scand 1962 56 249—257 — Whole body autoradiograms of mice injected intravenously with ( ) nicotine methyl C show an initial accumulation of radioactivity in the CNS This high concentration of nicotine and/or its metabolites in the brain disappears within 30 min to 1 hour Autoradiograms of cat brain sho a similar accumulation of radioactivity following i v injection of C -nicotine The distribution picture changes with time after injection Only one metabolite viz cotinine has been found to occur in the brains of mice and cats The possible existence of nicotine receptors in the brain is discussed

Following intravenous administration of C^{14} nicotine to mice a rapid accumulation of radioactivity in the brain was observed (SCHMITERLÖW and HANSSON 1962 HANSSON and SCHMITERLÖW 1962) The maximal concentration of radioactivity in the brain was reached within a very short time following which the radioactivity in the brain decreased rather quickly Preliminary investigations showed that nicotine was metabolized in the brain The identity of the metabolic compound formed was however not ascertained although the findings strongly suggested that the main metabolite was cotinine

Studies on the metabolism of nicotine in the brain are rather scarce WERLE and his co-workers (WERLE 1938 WERLE and MULLER 1941 WERLE and BECKER 1942 WERLE and USCHOLD 1948) in *in vitro*-experiments found a

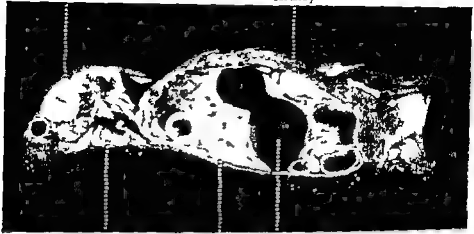

Fig. 1. Autoradiogram showing the distribution of radioactivity (light areas) in a mouse 5 minutes after intravenous injection of C nicotine. Sagittal section through the entire body of the animal. Note the accumulation of radioactivity in the brain.

detoxifying action of brain tissue only in two species, viz. man and rat. MILLER and LARSON (1953) also using the Warburg technique, were unable to find any nicotine detoxication by brain slices in mouse, rabbit, cat and dog.

As the uptake, distribution and metabolism of nicotine in the brain have not been very extensively studied the present study was undertaken in order to provide more information in this field.

It should be pointed out that a pre-requisite for this type of investigation is the use C^{14} nicotine with a very high specific activity. The high toxicity of nicotine makes this of paramount importance since only minute amounts of nicotine can be safely injected intravenously. Furthermore it should be emphasized that when performing tissue distribution studies using C^{14} labelled nicotine it is necessary to include investigations of nicotine metabolism. Both autoradiographic and radioassay techniques are based only on the presence of the radioactive indicator and do not discriminate between the parent substance and its possible metabolites.

Methods

Synthesis of ( ) nicotine methyl C^{14}

Nicotine methyl C^{14} was synthesized by methylating ( ) nornicotine using formaldehyde C^{14} in the presence of formic acid (McKENNIS *et al.* 1961) as described earlier by HANSSON and SCHMITERLÖW 1962. The radiochemical purity of the product was ascertained by performing autoradiography of paper chromatograms. The autoradiograms thus obtained showed only one radioactive spot, the R_f value of which corresponded to the R_f value of authentic nicotine. The specific activity of the ( ) nicotine methyl C^{14} was found to be 78 μC/mg.

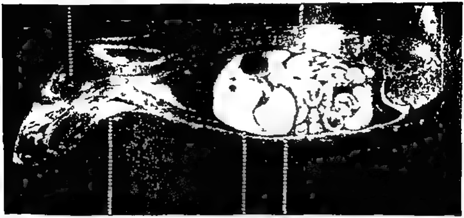

Fig 2 Autoradiogram showing the distribution of radioactivity (light areas) in a mouse 30 minutes after intravenous injection of C nicotine The concentration is high in salivary glands, stomach content liver and kidneys whereas the brain is almost free of radioactivity

Animal experiments

Two groups of 5 mice were injected slowly i v with a dose of 0 4 μg (corresponding to approximately 0 03 μC) C^{14} nicotine per g body weight The mice were sacrificed at 5 10 30 and 60 min and at 4 hours after the injection by immersing them in a mixture of acetone and dry ice Whole body autoradiography was then performed according to the method developed by ULLBERG (1954)

In order to study the metabolism of nicotine in the brain 8 mice were injected with the same dose of C^{14} nicotine as stated above and sacrificed after 30 min. The brains were immediately removed and homogenized in water Nicotine and the presumed metabolite cotinine were extracted according to HUCKER GILLETTE and BRODIE (1960) The metabolite was precipitated as the monopicrate according to BOWMAN TURNBULL and MCKENNIS (1959) after addition of carrier cotinine

Four kittens (body weight 0 5—0 6 kg) under pentobarbital anesthesia were injected i v with C^{14} nicotine and sacrificed by exsanguination at 5 15 30 and 60 min after the injection. The brain was removed and frozen on dry ice sectioned and autoradiographed The parts of the brain remaining after sectioning for autoradiography were homogenized in water and extracted at pH 9 with chloroform The chloroform extract was then used for paper chromatography The solvent system used was 0 5 N ammonia water 95 ethanol n butanol (1 1 4 v/v) and Whatman no 1 paper was used The Koenig positive spots were located with a spray of alcoholic p-aminobenzoic acid which was followed by treatment with vapors of cyanogen bromide The radioactive spots were detected by means of autoradiography of the chromatograms

In order to obtain quantitative radioactivity data from the Koenig positive zones the paper chromatograms were cut into pieces and the radioactivity in these pieces was counted directly in a Packard Tricarb liquid scintillation counter The same counting device was used to obtain quantitative radioassays of tissue pieces punched out from 20 μ thick sections of mice The scintillation solution was an ethanol toluene mixture (2 5) and contained diphenyloxazole as the scintillator

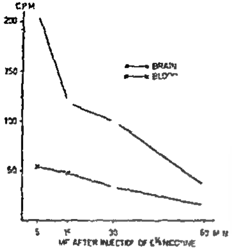

Fig 3 A comparison between the radioactivity in brain and blood of mice at different times after intravenous injection of C¹ nicotin For explanation see text.

Results

C^{14} nicotine and/or its metabolites in mice brain

The rapid accumulation of radioactivity in the brain following slow intravenous injection of C^{14} nicotine is shown in Fig 1 This is an autoradiogram from a mouse sacrificed 5 min after the beginning of the injection It is evident from this picture that there is a high concentration of radioactivity in the brain Fig 2 shows an autoradiogram from a mouse sacrificed 30 min after injection If these two pictures are compared it is obvious that the initial high concentration of radioactivity in the brain decreases fairly rapidly, the brain in Fig 2 showing only a low amount of radioactivity This is particularly apparent if the brain is compared with other organs such as liver stomach, intestines salivary glands or kidneys where the radioactivity is still high

In order to elucidate this time dependent variation in brain radioactivity the following radioassays were carried out. From 20 μ thick sections of the mice small pieces were punched out from the brain and from heart blood. The radioactivity in these pieces was measured in a liquid scintillation counter Fig 3 gives the results thus obtained It can be seen that the values (in cpm) for brain and blood are quite different five minutes after injection whereas the difference is almost negligible 60 min after injection.

Chloroform extracts of mouse brain subjected to paper chromatography showed only two main radioactive spots made evident by autoradiography One of these had an R_f value of 0 86 which corresponds to the R_f value of nicotine The R_f value of the other spot was 0 74 and thus corresponded to the R_f value of cotinine In order to ascertain whether this metabolite was really identical with cotinine we tried to isolate it by crystallization as the monopicrate This isolation was performed on brains from mice sacrificed 30 min

Table I The relative concentrations of n cotine and cotinine in the brain of mice at d fferent times after i v injection of C[14] nicotine

Mice no	Time after C[14] nicotine injection	radioactivity	
		Nicotine spot	Cotinine spot
7—8	5 min	72 9	27 1
5—6	15 min	80 9	19 1
3—4	30 min	24 9	75 1
1—2	1 hour	13 1	86 9

after the injection of C[14] nicotine Carrier cotinine was added The melting point of this monopicrate was found to be 102 —103 C which corresponded to the melting point of the cotinine monopicrate which was generously put at our disposal by Doctor Herbert McKennis Richmond Va USA The radioactivity of the monopicrate remained constant after recrystallization

The rate of conversion of nicotine into cotinine in the brain was followed by quantitative radioassays of the Koenig positive zones on chromatograms from brain extracts made from mice sacrificed at successive times after the intra venous injection of C[14] nicotine Table I gives the data thus obtained

As can be seen from the table nicotine is converted to cotinine at a fairly constant rate for 30 min and at the end of 60 min most of the nicotine has been converted to cotinine

Cotinine seems to be the only metabolite of nicotine that occurs in chloroform extracts of the brain No other Koenig positive spot could be found on the paper chromatograms nor could any other radioactive spot be detected on the autoradiograms of the paper chromatograms

Autoradiograms of cat brain including metabolic studies

Figs 4 and 5 show autoradiograms of brain sections at different levels from a cat sacrificed 5 min after i v injection of C[14] nicotine The highest level of activity is observed in the grey matter whereas the radioactivity is lower in the white matter It can also be observed that the nuclei of the diencephalon and the medulla oblongata have a tendency to concentrate radioactivity

In the brain of a cat sacrificed 15 min after the injection of C[14] nicotine there is still a distinct difference between white and grey matter as can be seen in Fig 6 In this autoradiogram it is of interest to note the fairly high concentration of radioactivity in the hippocampus particularly in certain areas presumably corresponding to the cell layers

After 30 min the picture has changed Fig 7 is an autoradiogram of a brain section from a cat sacrificed 30 min after the injection of C[14] nicotine The entire section now shows a lower amount of radioactivity and the radioactivity is also much more evenly distributed

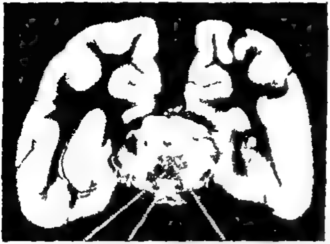

Fig 4 Autoradiogram of a transverse section of the brain (cat) at the anterior pontine level 5 minutes after intravenous injection of C^{14} nicotine (Light areas correspond to radioactivity) DBC = Decussatio brachiorum conjunctivorum IP= Nucleus interpeduncularis N III = Nucleus third nerve

Fig 5 Autoradiogram of a transverse section of the brain (cat) through the brain stem and cerebellum at a midcerebellar level 5 minutes after intravenous injection of C^{14} nicotine Light areas correspond to radioactivity
Coch = Nucleus nervi cochlearis Cul = Nucleus cuneatus lateralis Flp = Fasciculus longitudinalis posterior N V = Nucleus fifth nerve V Fifth nerve Pyr = Tractus pyramidalis

Chloroform extracts were made of cat brain after administration of C^{14} nicotine to the animal These extracts were run on paper and the chromatograms thus obtained showed only two Koenig positive spots Autoradiograms of these paper chromatograms revealed only two radioactive spots with the same R_f values as those found in mice brain viz one with an R_f value corresponding to nicotine and the other with an R_f value corresponding to cotinine A similar rapid conversion of nicotine to cotinine was found in cat brains as in mice brains Thus 30 min after injection of C^{14} nicotine almost all of the radioactivity found in the brain is present in cotinine and there are only traces of nicotine in the brain

Fig 6 Autoradiogram of a transverse section of the brain (cat) at the mesencephalic level through the red nucleus 15 minutes after intravenous injection of C[14] nicotine Light areas correspond to radioactivity
GC = Griseum centrale
GL = Corpus geniculatum laterale GM = Corpus geniculatum mediale Hip = Hippocampus NR = Nucleus ruber SN = Substantia nigra

Fig 7 Autoradiogram of a transverse section of the brain (cat) at the anterior hypothalamic level 30 minutes after intravenous injection of C[14] nicotine Light areas correspond to radioactivity

Discussion

The pharmacological effects of nicotine on the central nervous system have been extensively studied Some authors have observed a rapid onset and also a fairly rapid disappearance of the nicotine effect. LIBET and GERARD (1938) e g found that i v injection of 2 5 mg/kg nicotine in the cat blocked within 4 min tactile action potentials in the thalamus and somaesthetic radiations evoked by touching hairs of a contralateral leg This nicotine effect began to wear off in 20 min. GUALTIEROTTI (1952) studying spinal reflex activity in frogs also found a rapid onset of the nicotine effect as judged by the occurrence of large spontaneous discharges and a gradual disappearance of the effect in 20

30 min. These findings are quite in accord with our results, which clearly demonstrate the rapid accumulation of radioactivity in the brain of mice and cats after i. v. administration of C^{14} nicotine. Furthermore, we have also observed the gradual disappearance of radioactivity from the central nervous system indicating a rapid outflow of nicotine and/or its metabolites. This pattern of rapid uptake followed by gradual outflow seems to be peculiar to the brain: in whole body autoradiograms there is still a high level of radioactivity in other organs such as liver, stomach, intestines, kidneys, salivary glands etc. when the brain is almost free of radioactivity (see Fig. 2). The explanation of this phenomenon may be the following. In the chloroform extracts of brain we have found only one major metabolite of nicotine, viz. cotinine. The identification of this metabolite was made by paper chromatography and by isolation and crystallization of the metabolite from brain extracts using carrier cotinine. It is known that cotinine has very few, if any, pharmacological effects. McKennis (1960) states: "Pharmacological experiments have indicated that the metabolic oxidation of (-)-nicotine to (-)-cotinine leads to a dramatic loss of activity." Not only has cotinine a much lesser pharmacological action than nicotine, it may very well also be that cotinine has a lesser affinity for the tissues of the central nervous system. As cotinine is an oxidized form of nicotine it should be more polar and thus less fat soluble.

It has been repeatedly stated (see e. g. Brodie and Hogben 1957) that the entry of drugs into the central nervous system depends upon their lipid solubility. Since nicotine is lipid soluble it is easy to understand why it enters the brain. The actual accumulation of nicotine in the brain may be due to the presence of specific receptors or some other form of binding. According to Roth and Barlow (1961) an accumulation of a drug in the brain implies some form of binding or interaction between drug and tissue. It is tempting to assume that such receptors are localized in the nerve cells of the brain. From the autoradiograms of the cat brain it is obvious that the radioactivity is initially concentrated in the grey substance, whereas the white matter, with its higher lipid content, contains much less radioactivity. Our chromatographic studies have revealed that initially (5—15 min) the radioactivity present in the brain is due mainly to the C^{14} in nonmetabolized nicotine. Due to technical difficulties we have not yet been able to perform microautoradiographical studies of the brain. Such studies, which are in progress, might reveal whether or not radioactivity is actually concentrated in the nerve cells of the central nervous system. It should be mentioned that in some preliminary studies not yet published on the distribution of C^{14} nicotine in autonomic ganglia we have found a significant accumulation of radioactivity in the ganglion cells.

The nicotine receptors in the brain may not be able to serve as receptors for cotinine. This could explain the more diffuse distribution of radioactivity in the brain 30 minutes after the injection of C^{14} nicotine when the major part of the radioactivity is present as C^{14} in cotinine. According to Mayer, Maickel

and BRODIE (1960) even substances with rather low lipid solubility leave the cerebrospinal fluid readily, indicating a different kind of boundary from CSF to blood stream than from blood stream to CSF Although cotinine is less lipid soluble than nicotine there might thus be no difficulties for the brain to get rid of cotinine in a fairly short time either via the CSF or directly via the capillary walls

The authors wish to acknowledge the expert assistance of Docent G WIDMARK, Ph D Univers ty of Stockholm who synthesized the C[14]-nicotine The competent technical assistance of Mrs GUN PERSSON and Mrs ÅSA ASPLUND is gratefully acknowledged

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Acta physiol scand 1962 56 258—266

From the Department of Pharmacology University of Göteborg Sweden

Fluorimetric Determination of 3-O-Methylated Derivatives of Adrenaline and Noradrenaline in Tissues and Body Fluids

By

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Received 23 March 1962

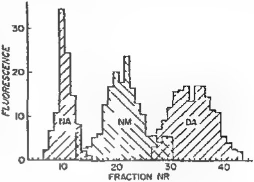
Abstract

HAGGENDAL J *Fluorimetric determination of 3-O methylated derivatives of adrenaline and noradrenaline in tissues and body fluids* Acta physiol scand 1962 *56* 258—266 — A method for the determination of small amounts of metanephrine and normetanephrine in tissues and body fluids is described The disturbing effect of adrenaline and noradrenaline is eliminated in two alternative ways 1 By differential oxidation and subsequent ion exchange chromatography 2 by separation on an ion exchange column Metanephrine and normetanephrine are determined in the eluate from the ion exchange column according to the principles of the trihydroxyindole method for the fluorimetric determination of adrenaline and noradrenaline Applications of the method on urine and brain tissue extracts are described

After AXELROD's (1957) discovery of metanephrine (M) and normetanephrine (NM) as metabolites of adrenaline (A) and noradrenaline (NA), the role played by the catechol O methyl transferase in the physiological inactivation of A and NA has been discussed According to AXELROD (1960) catechol O methyl transferase is the enzyme chiefly concerned with the metabolism and inactivation of A and NA

For the fluorimetric determination of M and NM the fairly weak fluorescence of the compounds in the ultraviolet region was utilized BERTLER, CARLSSON and ROSENGREN (1959) showed that the 3 O methylated derivatives of A and NA can be oxidized with iodine and then rearranged in alkali into strongly fluorescent compounds by utilizing the principles of the trihydroxyin

Fig 1 Separation by ion exchange cromatography of noradrenaline (NA) normetanephrine (NM) and dopamine (DA) extracted from the brains of mice treated with tranylcypromin Column Amberlite CG 120 type 2 H⁺ form length 35 mm diameter 4 2 mm Eluant 0.5 N HCl 1 ml per fraction The fluorescence obtained by treatment according to the THI method was read at th activating and fluorescent peaks of the respective compound (Ten fluorescence units correspond to 0 03 μg NA 0 13 μg NM and 0 16 μg DA per ml eluate)



dole (THI) method The determination is then made in mainly the same manner as the determination of A and NA according to BERTLER CARLSSON and ROSENGREN (1958) This method is more specific and sensitive than earlier methods

It is necessary to differentiate between the catechol amines and their respective 3 O methylated derivatives as the fluorescence characteristics of their fluorophores appear to be identical The differentiation can be performed by oxidation with iodine at different pH At a pH of about 5 only A and NA can be oxidized whereas also M and NM are oxidized at a pH of about 7 When M and NM are present in small amounts in relation to A and NA it is obvious however that the method will give uncertain values

Thus two other methods for the differentiation have been worked out

1 Selective destruction of A and NA followed by ion exchange chromatography

2 Total separation of A and NA from M and NM on ion exchange columns

Furthermore the method for fluorimetric determination of M and NM described by BERTLER *et al* (1959) has been modified

Differentiation of adrenaline and noradrenaline from metanephrine and normetanephrine

1 *Selective d struction* of A and NA can be performed with cyanide at a pH of about 6 5 At the subsequent column but not the oxidized A and NA will be adsorbed by the will thus contain only M and NM For details see C (1962) who have adopted the procedure for Instead of potassium ferricyanide iodine at a pH of 5 this laboratory The results however, were less tion of potassium ferricyanide seems to be much less

2 The technique for purification of of strong cation exchange resins (F

a total separation of A and NA from M and NM For the separation the resins Amberlite CG 120 type 2 and Dowex 50 X4 W X4 W X8 and W X12 have been used The elution is performed by means of hydrochloric acid The columns may be used in either sodium or hydrogen form.

Examples of this type of separation are given below

a) Analysis of brain extract

For the separation an Amberlite CG 120 type 2 column in hydrogen form length 35 mm diameter 4 2 mm was used Before the column was prepared the finest and coarsest particles of the resin were removed and the resin was washed with 2 N sodium hydroxide containing 1 per cent EDTA (disodium ethylenediamine tetra acetate) and 3 N hydrochloric acid The column was then washed as follows

1) Water to remove the acid
2) Sodium hydroxide 2 N 20 ml containing 1 per cent EDTA.
3) Water to remove the sodium hydroxide
4) Hydrochloric acid 2 N 20 ml
5) After washing with water to remove the acid the column was ready for use

After a finished separation the column could be used again if it was washed according to points 1—5 above

Tissue extracts were prepared by homogenizing brains of mice in 0 4 N perchloric acid The mice had been injected with tranylcypromin, 20 mg per kg i p 3 hours before they were killed

To the extract of 5 71 g brain) 20 mg EDTA and 4 mg ascorbic acid were added to improve recoveries The pH of the extract was then adjusted to about 6 (indicator paper) by adding 5 N potassium carbonate The solution was cooled and the precipitate spun down at 0 C Of the solution 10 ml were passed through the column All liquids were flowed through the resin bed at a rate of about 10 ml per hour After the sample 30 ml water and 15 ml 0 1 M phosphate buffer pH 6 5 were passed through The buffer was followed by 5 ml water The elution was then performed with 0 5 N hydrochloric acid All liquids except the last 5 ml water and the acid contained 0 1 per cent EDTA The eluate was collected in one ml fractions and every fraction was determined according to the principles of the THI method

Fig 1 shows the results Three separate peaks were obtained the first representing NA the second NM and the third DA DA was determined according to CARLSSON and WALDECK (1958)

b) Analysis of urine

Urine (as distinguished from brain) usually contains A and M besides NA and NM Furthermore the salt content is more fluctuating A suitable column was found to be Amberlite CG 120 type 2 length 50 mm diameter 4 2 mm. The column was used in sodium form After washing as above an 0 1 M phosphate buffer pH 6 5 passed through the column until the pH of

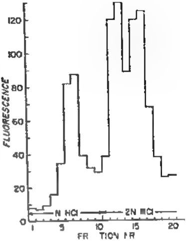


Fig 2 Separation of adrenaline (A) and noradrenaline (NA) from metanephrine (M) normetanephrine (NM) and dopamine (DA) in hydrolyzed urine to which 2 5 μg A NA M NM respectively and 10 μg DA had been added Column Amberlite CG 120 type 2 Na^+ form length 50 mm diameter 4 7 mm Eluant N and 2 N HCl 1 ml per fraction The fluorescence is read at the activating wavelength 285 and at the fluorescent wavelength 330 mμ (Fifty fluorescence units correspond to about 1 μg of each compound per ml eluate)

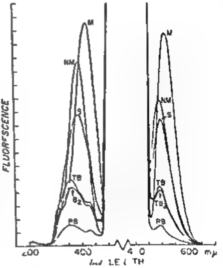


Fig 3 Activating and fluorescent spectra of an eluate (fraction II) of hydrolyzed urine when analyzed for metanephrine and normetanephrine as described in the text Left Activation spectrum Fluorescent wavelength 510 mμ Right Fluorescent spectrum. Activating wavelength 420 mμ M Metanephrine standard 0 061 μg per ml NM Normetanephrine standard 0 061 μg per ml S Sample and the tissue blanks are read at about three times higher sensitivity of the apparatus than the standards TB_1 Tissue blank without oxidation TB Tissue blank faded RB Reagent blank read at the same sensitivity as the standards

the effluent was about 6 5 After washing with 10 ml water the column was ready for use

To the sample 5—10 ml urine (may be hydrolyzed in N perchloric acid) 20 mg EDTA and 4 mg ascorbic acid were added The pH of the sample was adjusted to about 6 with potassium carbonate The sample was centrifuged at 0 C and then passed through the column. After washing with 30 ml water 15 ml 0 1 M phosphate buffer pH 6 5 and 10 ml water the elusion was performed with hydrochloric acid The first 30 ml water and the buffer contained

0 1 per cent EDTA All the liquids were passed through the columns at the rate of about 10 ml per hour

Fig 2 shows the separation of a sample of 5 ml urine which had been boiled for 20 min in N perchloric acid and to which 2 5 mμ A, NA M, and NM and 10 mμ DA had been added The fluorescence of the eluate was read ml by ml at 330 mμ with the activating wavelength at 285 mμ (all wavelengths given in this paper are uncorrected instrumental values, Aminco-Bowman spectrophotofluorometer) All these compounds have the same fluorescence characteristics

The following results were obtained

a) The first 2 ml eluate (N hydrochloric acid) contained only interfering substances and were discarded

b) The following 8 ml N hydrochloric acid (fraction I) contained the A and the NA The amount of M and NM in this fraction could be neglected

c) The elution was then continued with 10 ml 2 N hydrochloric acid (fraction II), which contained the M, NM and the DA, and only negligible residues of A and NA

In a parallel experiment on the same hydrolyzed urine but without any additions of amines fraction II was analyzed according to the THI method The values are given in Table II Spectra are shown in Fig 3 The amounts of A and NA in fraction II were found to be negligible

The column separation has been of such a good reproducibility that it has been possible to follow the scheme described above (a c) in the assay of most urines When the recoveries of added M and NM have been less than 80 per cent the reason usually has been an unsuccessful separation, because of *e g* a highly concentrated urine or incorrect flow rates Destruction of M and NM during the column procedure does not seem to be of any importance During the column procedure M and NM were more stable than A, NA and DA

By lengthening the column it was possible to separate DA from the M NM fraction Further lengthening of the column will give a complete separation of NA A, NM, M and DA in one run (HÄGGENDAL 1962) It may be useful for identification purposes and for determination of small amounts of *e g* M in the presence of large amount of NM which has been difficult with previous methods

Since assay of both A, NA, and DA and their 3 O methylated derivatives often is necessary in order to obtain a reasonably complete picture of the catechol amine metabolism the separation procedure in one run on a suitable column may be time saving

Fluorimetric determination of M and NM

Procedure

1 The pH of the eluate is adjusted to about 6 (indicator paper) with 5 N potassium carbonate

Table I Differential estimation of metanephrine (M) and normetanephrine (NM) in pure solutions Readings were performed at the wavelength combinations (activating wavelength/fluorescent wavelength) 370/480 mµ and 440/570 mµ

Added µg to sample[1]		Found µg in sample (Mean values ± S D)	
M	NM	M	NM
0 190	0 010	0 204 ± 0 0037	0 011 ± 0 0030
0 150	0 050	0 154 ± 0 0071	0 050 ± 0 0074
0 100	0 100	0 103 ± 0 0047	0 101 ± 0 0030
0 050	0 150	0 053 ± 0 0075	0 147 ± 0 0062
0 010	0 190	0 016 ± 0 0015	0 178 ± 0 0077

[1] Total volume of the sample 3 3 ml

2 Immediately after the pH adjustment 1 0 ml *sample* is added to 0 5 ml buffer (0 6 M citrate and 1 M phosphate buffer equal parts pH 6 5) and 0 2 ml water

3 The oxidation is performed with 0 10 ml of 0 02 M iodine solution

4 After 3 min 0 5 ml of 5 per cent sodium sulfite solution containing [illegible] per cent EDTA is added After 30 sec 0 5 ml of 5 N sodium hydroxide is added

5 After 5 min the sample is acidified with 0 5 ml of 10 N acetic acid

6 After about 20 min the fluorescence of the sample is read at two wave length combinations *e g* at the fluorescent wave lengths 485 and 510 mµ with the activating wave lengths 390 and 440 mµ, respectively

7 *Standards* containing 0 2 µg M and 0 2 µg NM as well as *internal standards* *i e* samples to which known amounts of M and NM have been added are carried through the procedure along with the sample and the blanks

8 Two types of *tissue blanks* may be used

a *a blank treated as the sample except that iodine is omitted (T B without oxidation)*

b a blank treated as the sample except that the sulfite EDTA solution is not added until 10 min after the sodium hydroxide (T B faded)

9 A *reagent blank* (R B) containing all the reagents but no sample or standard is also carried through the procedure

10 The absence of A and NA in the eluate is checked by oxidation with potassium ferricyanide according to the THI method

Results of differential estimations of M and NM in pure solutions are given in Table I The coefficient of variation was between 1 3 per cent and 4 8 per cent if the M or the NM in the sample (3 3 ml) not were less than 0 015 µg per ml

Table II Analysis of M and NM in 5 ml human urine hydrolyzed for 20 minutes in N perchloric acid

	Sample	Tissue blank without oxi dation	NM intern. stand	M intern. stand.	NM stand.	M stand	Reagent blank
NM[1] ml	—	—	0 2	—	0 2	—	—
M[1] ml	—	—	—	0 2	—	0.2	—
Eluate pH 6 ml	1 0	1 0	1 0	1 0	—	—	—
Buffer ml	0 5	0 5	0 5	0 5	0 5	0 5	0 5
H_2O ml	0 2	0 3	—	—	1 0	1 0	1 2
I Iodine 0 02 N ml	0 1	—	0 1	0 1	0 1	0 1	0 1
II Na_2SO_3 + EDTA ml (added after 3 min after I)	0 5	0 5	0 5	0 5	0 5	0 5	0 5
III NaOH 5 N ml (added after 30 sec after II)	0 5	0 5	0 5	0 5	0 5	0 5	0 5
IV HAc 10 N ml (added after 5 min after III)	0 5	0 5	0 5	0 5	0 5	0 5	0 5
Total volume ml	3 3	3 3	3 3	3 3	3 3	3 3	3.3
Fluorescence intensity read at 370/480 mμ	20	11	63	35	[2]50	24	5 8
Fluorescence intensity read at 440/520 mμ	8 0	4 2	16	51	9 3	[3]50	2 8

[1] Containing 1 0 μg DL 3 O methylated catechol amine hydrochloride per ml

[2] The normetanephrine standard was set to 50

[3] The metanephrine standard was set to 50

The metanephrine (0 2 x mμ) and normetanephrine (0 2 y mμ) in the sample are calculated by solving the equations

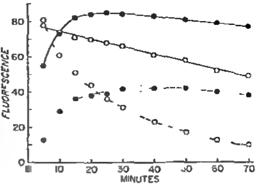
$$(35 - 0)x + (63 - 20)y = 20 - 11 \quad \text{(Readings at 370/480 m}\mu\text{)}$$
$$(51 - 8)x + (16 - 4)y = 8\,0 - 4\,2 \quad \text{(Readings at 440/520 m}\mu\text{)}$$
$$x = 0\,03$$
$$y = 0\,20$$

The total values of metanephrine and normetanephrine in the hydrolyzed 24 hr urine were 33 μg and 220 μg respectively

An example of analysis of an eluate from urine is given in Table II In so far as this method differs from the method of BERTLER *et al* (1959) the different steps of the procedure will be discussed below

Ad 1 and 2 The intensity of the fluorescence is known to be dependent on the pH, at which the oxidation is performed Using the present buffer pH 6 5 was found to be optimal The intensity was higher if citrate was added to the buffer Thus a phosphate citrate buffer pH 6 5, is used instead of a phosphate buffer, pH 7 15

Fig 4 Effect of different antioxidants on the stability of the fluorophores of metanephrine (M) and normetanephrine (NM) Circles M Dots NM Broken lines ascorbic acid solution Continuous lines EDTA — sulfite solution



Ad 3 The amount of iodine is increased as this was found to give a somewhat higher intensity In pure solutions the presence of DA resulted in a loss of the intensity of the fluorescence of M and NM if the amount of iodine was low

Ad 4 To stop the oxidation the EDTA sulfite solution is used instead of ascorbic acid The fluorescence of M will be more stable than when ascorbic acid is used (Fig 4) The figure also illustrates that with the EDTA sulfite solution a higher fluorescence intensity is obtained

No useful results were obtained by adding metal ions in order to catalyze the oxidation Cu^{++} ions were found to accelerate the decomposition of the fluorophores (as they do for A and NA)

Ad 5 By using a stronger acetic acid solution the intensity of the fluorescence will be higher Hydrochloric acid instead of acetic acid yielded a lower intensity

Ad 6 For differentiation between M and NM the differences in fluorescence characteristics can be utilized (BERTLER *et al* 1959) The best reproducibility seemed to be obtained if the differences in both the activation and the fluorescence characteristics of the compound were utilized Thus readings are performed at the activating wavelength 390 mμ with the fluorescence at 485 mμ and at the activating 440 mμ with the fluorescence at 510 mμ The calculations are done as for A and NA

Ad 7 The sample will contain much more salt than the standards because it is a neutralized eluate (as a rule 1 or 2 N hydrochloric acid adjusted by 5 N potassium carbonate) the standards of M and NM are kept in 0 01 N hydrochloric acid The same amount of M or NM will give different intensity if the oxidation is performed with or without addition of salt However the differences are small when this citrate phosphate buffer pH 6 5 is used Most problems of this type are eliminated if internal standards are used

Ad 8 The two types of blanks give about the same readings Larger differences may be observed when ascorbic acid is used as an antioxidant

I wish to thank Miss MARGIT LINDQVIST for her help and interest For technical assistance I am indebted to Mrs INGEGERD GRUNDSTROM and Miss BIRGITTA Lundgren This work was supported by grants from the Medical Faculty of Goteborg and by the Air Force Office of Scientific Research of the Air Research and Development Command United States Air Force

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Lactate and Pyruvate Formation and Oxygen Utilization in the Human Forearm Muscles During Work of High Intensity and Varying Duration

By

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Abstract

PERNOW B and J WAHREN *Lactate and pyruvate formation and oxygen utilization in the human forearm muscles during work of high intensity and varying duration* Acta physiol scand 1962 *56* 267—285 — The work was performed with a hand ergometer the intensity of work being varied with respect to frequency and load Blood samples were obtained through catheters inserted in the brachial artery and in deep and superficial forearm veins The deep veins of the forearm contain fairly pure muscular blood during and immediately after work, whereas at rest there is an admixture of blood from the hand At rest a significant difference was present between the arterial and deep venous blood with respect to oxygen saturation lactate and pyruvate concentration and pH During work of about 4 min duration a significant rise occurred in both arterial and venous lactate and pyruvate The highest venous lactate concentration was usually noted at the end of work while pyruvate did not reach its maximum until 5 min after work The resting levels were regained about 45 min later The oxygen saturation of the venous blood fell from a mean 56 6 to 23 9 per cent In connection with work of high intensity but very short duration (5—30 sec) a marked increase was observed in venous lactate and pyruvate while very small changes were seen in arterial blood

Changes in the concentration of lactate and pyruvate in arterial blood in connection with muscular work have been the subject of extensive studies (e g STROM and LUNDIN 1947 ASMUSSEN 1950 HUCKABEE 1958 1959) The rise in lactate and change in the lactate pyruvate ratio during work have been generally accepted as an expression of the existence of anaerobic conditions The opinion varies however regarding the degree of anaerobic metabolism at different work intensities Thus HUCKABEE (1958) stated that an anaerobic component is present at every grade of muscular work, even a light one

CHRISTENSEN, HEDMAN and SALTIN (1960) found however that heavy work carried out for extremely short periods (5—15 sec) was unaccompanied by any demonstrable rise in lactate concentration in the arterial blood. They interpreted this observation to imply that such brief work takes place under *completely aerobic conditions, probably with myoglobin as an oxygen store.*

It has been stressed in recent years, particularly by HUCKABEE (1958), that the lactate pyruvate ratio is a better index of anaerobic conditions than the blood concentration of lactate alone since the latter can evidently be elevated *also in states where hypoxia is lacking.* This is evident e.g. from the fact that after muscular work the lactate concentration does not reach the resting level until about 45 min whereas the lactate pyruvate ratio is rapidly normalized (CARLSON and PERNOW 1961).

As shown by COOPER, EDHOLM and MOTTRAM (1955) the flexor muscles of the forearm seem to comprise a muscle group which, in man, is most suitable for metabolic studies *in vivo.* It is possible, via the median cubital vein, to advance a catheter into one of the deep veins of the forearm, i.e. the ulnar, interosseous or radial vein. Samples from the interosseous vein seem to consist of pure muscular blood, whereas those from the other two veins contain, under resting conditions, small amounts of blood from the hand (MOTTRAM 1955, COLES *et al* 1958). This technique has since been used among others by RODDIE, SHEPHERD and WHELAN (1956), MONOD *et al* (1960 and 1961) and ANDRES, CADER and ZIERLER (1961). MONOD *et al* (1960) studied the lactate concentration of deep venous blood during work and found that heavy work of only a few minutes duration gave a marked increase in lactate while light work could be performed during long periods without any rise in lactate.

In the present investigation the lactate and pyruvate formation and the oxygen utilization in the forearm muscles were studied in healthy subjects at rest and during work using a catheterization technique similar to that described by MOTTRAM (1955). The ratio of lactate to pyruvate expressed according to HUCKABEE (1959) as excess of lactate (XL) was used as an index of the local anaerobic metabolism.

Case material

The case material consisted of 31 healthy males. 10 of them were welltrained firemen, all active athletes. The remainder were students. The mean age of the whole material was 27 years (21—40), the weight 70.6 kg (63.5—85.0) and height 179 cm (167—187).

Procedure

The subjects came fasting to the laboratory in the morning. Work was performed with a hand ergometer[1] with the subject in supine position and with the arm horizontal. The work consisted of squeezing two parallel bars together in the hand. The distance between them in the resting position was adapted for each subject before starting work.

[1] The hand ergometer was manufactured by AB PIAB Stockholm.

and was not changed in the course of the experiment The load on the handle consisted of a calibrated spring and the work per contraction was varied by allowing the spring to be shortened to a varying degree during work. The load on the hand ergometer could in this way be varied from 25 to 150 kpmm/contraction The uniformity of the squeezes was checked by a signal system which was actuated as soon as the spring was compressed to a certain position Unless otherwise stated the contractions were made rhythmically to a metronome which gave 60 signals per minute

Teflon catheters were introduced percutaneously into the brachial artery in the proximal direction into the median cubital vein and in some cases into a superficial vein of the forearm in the distal direction The median cubital vein was punctured in the anticubital fossa and the catheter passed through a deep communicating vein 6—8 cm downstream until its tip was no longer palpable The blood obtained through this catheter is called deep venous blood In 4 cases the tip of the deep catheter was still palpable after being advanced more than 10 cm In these cases there was no significant difference between the oxygen saturation of the samples taken from the two catheters these cases were therefore excluded from the material

Analytical methods

Blood lactic acid was determined with Strom s (1949) modification of the colorimetric method of Barker and Summerson (1941) and pyruvic acid according to the method of Friedemann and Haugen (1943) as modified by Huckabee (1956) For the analyses in connection with work the precipitation with trichloroacetic acid was made within 15—20 sec Resting values obtained for lactate by this technique was on an average 15 per cent higher and for pyruvate 15 per cent lower than those obtained when dripping the blood directly from the catheter into weighed tubes containing the trichloroacetic acid This finding is in agreement with what has been reported by HUCKABEE (1956) The values given for lactate and pyruvate at rest (Table I) were obtained by the last mentioned technique No corrections were made for changes in lactate and pyruvate due to variations in the hematocrit during work

Excess of lactate (XL) was calculated from the formula $XL = L_v - L_a - L_a/P_a \, (P_v - P_a)$ where L_a and L_v are the lactate concentration in arterial and venous blood respectively and P_a and P_v the corresponding pyruvate concentrations (HUCKABEE 1959)

Samples for determination of oxygen saturation and oxygen content were drawn simultaneously from arterial and venous catheters into 10 ml syringes the dead spaces of which were filled with 1 per cent heparin solution The oxygen saturation and hemoglobin concentration were determined spectrophotometrically by a slight modification of the method described by DRABKIN (1958) The oxygen content was calculated from the oxygen saturation and oxygen capacity The syringes were always filled with the same volume of blood and no correction was made for the heparin filled dead space

The pH of whole blood was measured at 37 C by a glass electrode and a potentiometric pH meter using phosphate buffer as standard No temperature correction was made

Expressed as the coefficient of variation for a single observation calculated from duplicate determinations on one blood sample the error of the method in the lactate determinations was 4 4 per cent ($n = 194$) in the measuring range 0—3 mM/l and 2 1 per cent ($n = 50$) at concentrations between 3 0 and 11 mM/l The error of the method for pyruvate determinations was 3 1 per cent ($n = 50$) in the 0—0 150 mM/l range 1 3 per cent ($n = 50$) at concentrations of 0 150—0 300 and 0 9 per cent ($n = 50$) at concentrations of 0 300—0 450 mM/l The coefficient of variation for the determinations of oxygen saturation was 0 73 per cent ($n = 102$) at saturations ranging from 10 to 100 per cent and for hemoglobin 0 ? per cent The error of the method for pH determinations was 0 04 per cent

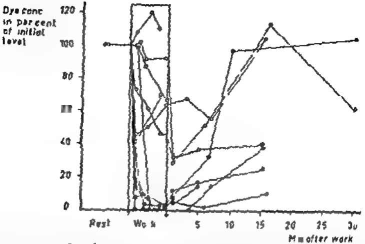

Fig 1 Concentration of cardio-green in superfical (open circles) and deep venous blood (filled circles) in per cent of the initial level The dye was continuously infused into the radial artery in 7 cases. The blood was withdrawn repeatedly from both veins before during and after work of approximately 5 min duration

The heart rate was calculated from the ECG

The room temperature ranged from 18 to 21 C on different occasions during the experiments and no arrangement was made to keep it constant

Results

CONTROL STUDIES

0 01 per cent indocyanine (Cardio green) solution was infused at a constant rate through a teflon catheter introduced into the radial artery in the distal direction During infusion repeated samples were taken simultaneously from the superficial and deep veins The colour concentration in plasma was read spectrophotometrically at 810 mμ At rest colour was demonstrated in blood from both the superficial and deep veins in 7 of 10 cases In the remaining 3 cases the oxygen saturation was unchanged before and after application of a cuff around the wrist indicating that the tip of the deep venous catheter was lying in the interosseous vein When the dye was infused during work the concentration of dye was always markedly reduced in deep venous blood (Fig 1) The duration of the work was a mean 5 min 13 sec and the work intensity 7 5 kpm/min The work was performed with 60 contractions per min At the end of work no dye at all could be detected in 4 cases while in the remaining 3 cases the dye concentrations did not exceed 3 per cent of the initial level After work the dye concentration of the deep venous blood increased successively but still 15 min after the end of work the dye concentrations were in most cases below 40 per cent of the initial values The changes in the dye concentrations of the superficial vein were irregular but in most cases a decrease was observed during work, which however, never exceeded about 50 per cent of the resting

Table I Resting values for oxygen saturation oxygen content lactate and pyruvate concentration in blood samples from the brachial artery a deep vein and a superficial vein

		Oxygen saturation per cent	Oxygen content ml/100 ml	Lactate mM/l	Pyruvate mM/l
Brachial artery	M	96.5	16.5	0.61	0.097
	SEM	0.3	0.22	0.04	0.006
	SD	1.8	1.18	0.14	0.023
	n	28	28	13	13
Deep vein	M	55.9	9.3	0.76	0.109
	SEM	2.4	0.46	0.06	0.008
	SD	13.2	2.53	0.20	0.028
	n	31	31	[illegible]	13
Superficial vein	M	76.0	12.8	1.08	0.114
	SEM	3.1	0.58	0.06	0.008
	SD	11.6	2.09	0.23	0.022
	n	13	13	13	9

M = mean SEM = standard error of mean SD = standard deviation n = number of observations

level In these studies the dye infusion rate (1.5—2.5 ml/min) and the dye concentration of the infusion fluid was so chosen that adequate light absorption was secured even with a 50 fold increase in blood flow compared to the flow at rest

In order to ascertain whether the catheter in the brachial artery limited the blood flow to any significant degree during work 5 subjects were instructed to perform the same type of work before and after insertion of the catheter No significant difference was found between the oxygen saturation of corresponding samples of blood from the deep forearm vein ($P < 0.3$)

METABOLIC STUDIES

At rest (Table I)

Lactate concentration The mean concentration in arterial blood was 0.61 ± 0.14 mM/l[1] in blood from a deep forearm vein 0.76 ± 0.20 mM/l and in superficial venous blood 1.08 ± 0.23 mM/l The arterio-venous (AV) differences are highly significant ($P < 0.001$) and the difference in concentration between superficial and deep venous blood is probably significant ($P < 0.02$)

Pyruvate concentration In arterial blood a mean concentration of 0.097 ± 0.023 mM/l was recorded The corresponding figure for deep venous blood was 0.109 ± 0.028 mM/l and for blood from a superficial vein 0.114 ± 0.022 mM/l

[1] All values are given as mean ± standard deviation.

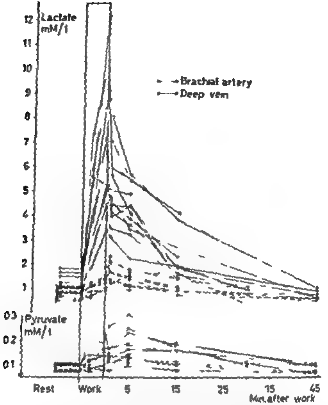

Fig 2 Lactate and pyruvate concentrations of arterial and deep venous blood at rest during and after work of 2—7 min duration. Filled circles and broken lines indicate arterial blood while open circles and straight lines denote deep venous blood. During work blood was in most cases drawn after 2 min and in all cases at the end of work. Although the duration of work varied all end of work values are indicated on the same line for comparison

The AV differences are significant ($P < 0.01$) as well as the inter venous difference ($P < 0.001$)

These values for lactate and pyruvate were all obtained by the method of rapid precipitation whereas values in the following were registered in blood samples handled with pipetting technique

Oxygen saturation The mean arterial oxygen saturation was 96.5 ± 1.8 per cent. A mean value of 55.9 ± 13.2 per cent was measured in blood from the deep forearm vein while the corresponding value for the superficial vein was 76.0 ± 11.6 per cent. The difference between the oxygen saturation of deep and superficial venous blood is significant ($P < 0.01$)

Work of 2—7 min duration (Fig 2 3 4 Table II)

This type of work was performed in 17 cases at a mean intensity of 7.1 ± 1.0 kpm/min and a rate of 60 contractions/min. The subject continued to work until maximal exhaustion and the duration ranged from 2 min 15 sec to 7 min

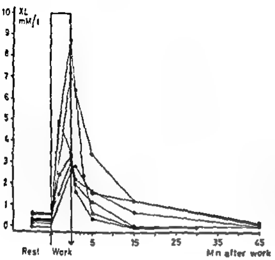

Fig 3 Formation of "excess of lactate" (XL) in the forearm in connection with work of 2—7 min duration. All end of work values are indicated on the same line although the duration of work varied

mean 4 min 16 sec and the range of the total work performed was 17 0—41 6 kpm

The *lactate concentration* in the deep venous blood rose from a mean 0 98 ± 0 44 mM/l at rest to 4 41 ± 2 03 mM/l measured 2 min after starting work. At the end of work it had reached a mean of 6 42 ± 2 56 mM/l Both these changes from the resting level are significant ($P < 0.005$ and $P < 0.001$ respectively) In samples from the superficial vein the lactate concentration rose from a mean 1 15 ± 0 22 mM/l at rest to 4 25 ± 0 82 at the end of work In every case this rise was smaller than that recorded simultaneously in the deep vein of the forearm The difference between the lactate concentration of samples from the deep and the superficial vein at the end of work was a mean 3 63 mM/l ($P < 0.05$) During the same work the arterial lactate rose from 0 81 ± 0 26 mM/l at rest to 1 33 ± 0 31 at the end of work This rise is significant ($P < 0.001$) as is the difference in lactate between arterial and deep or superficial venous blood during work ($P < 0.001$)

After work, the lactate concentration fell rapidly in the venous blood and in all but two cases lower values were recorded as soon as 1 min after it as compared to those at the end of work In the two cases mentioned the concentrations were essentially unchanged during the first min after work A successive decrease in lactate concentration took place thereafter and the initial level was reached 30—45 min after the end of work The lactate concentration of the arterial blood on the contrary continued to rise after the end of work in 7 of 8 cases One minute after work levels were reached that were significantly higher than at the end of work ($P < 0.01$) Thereafter the concentration gradually decreased and had fallen to the resting level 30—45 min after work.

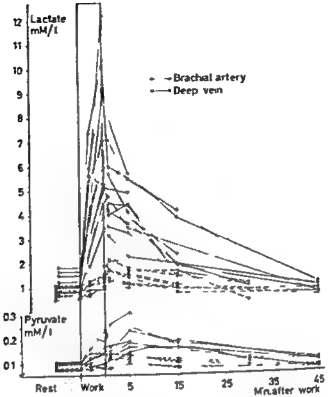

Fig 2 Lactate and pyruvate concentrations of arterial and deep venous blood at rest during and after work of 2—7 min duration. Filled circles and broken lines indicate arterial blood while open circles and straight lines denote deep venous blood. During work blood was in most cases drawn after 2 min and in all cases at the end of work. Although the duration of work varied, all end of work values are indicated on the same line for comparison.

The AV differences are significant ($P < 0.01$) as well as the inter venous difference ($P < 0.001$)

These values for lactate and pyruvate were all obtained by the method of rapid precipitation whereas values in the following were registered in blood samples handled with pipetting technique

Oxygen saturation The mean arterial oxygen saturation was 96 5 ± 1 8 per cent. A mean value of 55 9 ± 13 2 per cent was measured in blood from the deep forearm vein while the corresponding value for the superficial vein was 76 [illegible] ± 11 6 per cent The difference between the oxygen saturation of deep and superficial venous blood is significant ($P < 0.01$)

Work of 2—7 min duration (Fig 2 3 4 Table II)

This type of work was performed in 17 cases at a mean intensity of 7 1 ± 1 0 kpm/min and a rate of 60 contractions/min The subject continued to work until maximal exhaustion and the duration ranged from 2 min 15 sec to 7 min

1 min after work		5 min after work		15 min after work		30—45 min after work	
A	DV	A	DV	A	DV	A	DV
	85 1	96 7	87.5	97 7	66.9	96 9	69 1
	1 46		1 64		9 14		5 87
	3 27		4 35		20 45		[illegible] 40
	5	4	7	3	5	3	7
	0 005		0 01		0 1		0 1
1 75	5 01	1 56	4 32	1 26	2 47	0 83	1 14
0 15	0 46	0 14	0 29	0 13	0 36	0 11	0 17
0 49	1 53	0 43	1 03	0 37	1 13	0 34	0 48
8	11	10	13	8	10	9	8
0 001	0 001	0 001	0 001	0 005	0 005	0 6	0 3
0 103	0 156	0 111	0 213	0 095	0 176	0 081	0 096
0 010	0 007	0 009	0 008	0 077	0 006	0 006	0 006
0 095	0 017	0 023	0 020	0 05	0 014	0 014	0 015
6	6	6	6	5	5	6	6
0 9	0 025	0 005	0 001	0 3	0 001	0.5	0 02
2 90		1.50		0 24		0 03	
0 71		0 44		0 03		0 05	
1 75		1 08		0 73		0 10	
6		6		5		5	
0 025		0 05		0 99		0.2	
	7.286		7 330	7 425	7 393		
					0 004		
					0 010		
	4		2	4	5		
					0 1		
63 7							
2 4							
6.5							
7							
0 7							

icance of difference from the corresponding resting value Other symbols as in Table I

The *excess of lactate (XL)* was calculated from the differences in lactate and pyruvate concentration in arterial and deep venous blood It indicates the formation of lactate which is not due to changes in pyruvate concentration XL increased during work parallel to the rise in lactate concentration of the venous blood Thus a marked rise in XL was observed during work *i e* from a mean 0 31 $\pm$ 0 21 to 4 41 $\pm$ 2 50 mM/l at the end of work ($P < 0\ 001$) A return to the initial level occurred rapidly and in all but one case a pronounced decrease was recorded as soon as 1 min after the end of work. The XL factor was restored to the initial level in a shorter time than that required for

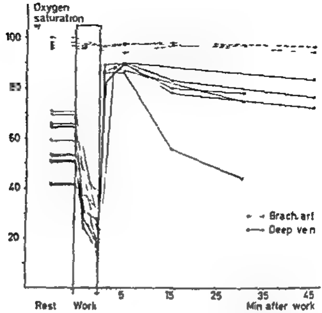

Fig 4 Oxygen saturation in arterial and deep venous blood at rest, during and after work of 2—7 min duration All values obtained at the end of work are indicated on the same line Symbols as in Fig 2

restitution of the lactate and pyruvate concentrations to the resting level

The *pH* of the blood from the deep forearm vein was a mean 7 386 $\pm$ 0 015 at rest the corresponding value in the arterial blood being 7 437 $\pm$ 0 012 The AV pH difference is significant ($P < 0\,001$) At the end of work, the pH of the blood from the deep vein had fallen to 7 191 $\pm$ 0 017 ($P < 0\,001$) The lowest value recorded was 7 100 After work the pH rose successively and had reached the resting level 15 min later The pH of the arterial blood did not change during or after work

The *oxygen saturation* at rest was a mean 56 6 $\pm$ 10 4 per cent in samples from the deep forearm vein After two min of work it had fallen to 31 8 $\pm$ 6 5 per cent ($P < 0\,001$) and at the end of work to 23 9 $\pm$ 8 4 per cent *One minute after the end of work, the oxygen saturation had risen* to levels higher than the corresponding resting values ($P < 0\,005$) After 30—45 min the oxygen saturation was still higher than before work These values refer to the case material as a whole In a comparison between the firemen and the students the former were found at the end of work to have reached values that were probably significantly lower than those in the latter group ($P < 0\,05$) In no other respect was any difference obtained between the two groups of volunteers The total work performed was of the same order of magnitude in both groups and the lactate and pyruvate production during work was also the same

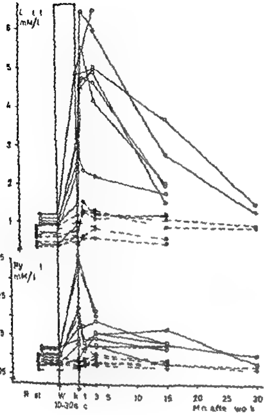

Fig 5 Lactate and pyruvate concentrations of arterial and deep venous blood of the forearm in connection with work of 10—30 sec duration. Symbols as in Fig 2

In blood from the superficial vein the oxygen saturation was a mean 72 3 ± 11 3 per cent at rest At the end of work it had fallen to 39 7 ± 8 3 per cent ($P < 0\ 05$) This decrease in oxygen saturation was smaller than in blood from the deep vein ($P < 0\ 001$)

The oxygen saturation of the arterial blood was a mean 96 6 ± 1 6 per cent at rest and did not change significantly during or after work

The *hemoglobin concentration* in blood from the deep forearm vein was a mean 13 34 ± 0 69 g/100 ml at rest At the end of work it had risen to 14 24 ± 0 74 g/100 ml ($P < 0\ 001$) In blood from the superficial vein the hemoglobin concentration rose from a mean 13 40 ± 0 69 g/100 ml at rest to 14 40 ± 0 63 g/100 ml at the end of work ($P < 0\ 001$) The arterial hemoglobin concentration was unchanged during and after work

The *heart rate* was a mean 64 1 ± 6 9 beats/min at rest After two min of work it had risen to 80 0 ± 8 0/min and at the end had reached a mean 97 7 ± 13 7/min ($P < 0\ 001$) The heart rate returned quickly to the resting level and restitution had occurred after 1 min in every case

Table III Some data obtained in connection with forearm exercise of 10—30 sec duration

		Rest		End of work	
		A	DV	A	DV
Oxygen saturation per cent	M	96 9	59 2	96 9	39 2
	SEM	0 7	6 2	0 3	5 5
	SD	2 1	17 5	0 7	15 4
	n	8	8	5	8
	P				0 025
Lactate mM/l	M	0 61	0 91	0 71	2 87
	SEM	0 07	0 09	0 03	0 50
	SD	0 20	0 25	0 27	1 42
	n	8	8	8	8
	P			0.2	0 001
Pyruvate mM/l	M	0 082	0 096	0 082	0 206
	SEM	0 004	0 006	0 008	0 011
	SD	0 013	0 017	0 023	0 031
	n	8	8	8	8
	P			0 95	0 01
ΔL mM/l	M	0 17		1 02	
	SEM	0 07		0 06	
	SD	0 20		0 18	
	n	8		8	
	P			0 01	
Heart rate	M	65 7		105	
	SEM	4 1		2 7	
	SD	10 8		7 3	
	n	7		7	
	P			0 001	

Symbols as in Table II

Work of 10—30 sec duration (Fig 5 Table III)

The effect of muscular work during 10—30 sec periods was studied in 8 subjects The average total work performed was 9 9 ± 2 7 kpm In these experiments as many contractions as possible were made during the work period giving a mean work intensity of 35 3 ± 13 5 kpm/min

The *lactate concentration* in blood from the deep vein rose significantly during this type of work from a mean 0 91 ± 0 25 mM/l to 2 87 ± 1 42 mM/l (P < 0 001) One and 3 min after the end of work the concentration had risen still further In the arterial blood the lactate concentration was essentially unchanged at the end of work but a tendency towards a rise was recorded 3 min later (0 025 < P < 0 05)

The *pyruvate concentration* in blood from the deep forearm vein had risen significantly at the end of work from a mean 0 096 ± 0 017 mM/l at rest to

1 min after work		3 min after work		15 min after work		30 min after work	
A	DV	A	DV	A	DV	A	DV
	81.6		83.4		72.6		
	1.8		0.8				
	4.1		1.8				
	5		5		4		
	0.025		0.05				
1.10	4.71	1.11	4.48	0.85	2.24	0.83	1.33
0.16	0.56	0.14	0.14	0.13	0.33		
0.40	1.37	0.39	0.40	0.33	0.81		
6	6	8	8	6	6	5	2
0.1	0.005	0.05	0.005	0.1	0.005		
0.080	0.133	[illegible]	0.163	0.080	0.126	0.066	0.09[illegible]
0.002	0.011	0.004	0.004	0.003	0.013		
0.005	0.025	0.011	0.011	0.007	0.031		
6	5	8	8	6	6	2	[illegible]
0.95	0.005	0.05	0.001	0.2	0.1		
3.50		2.90		0.50		0.16	
0.43		0.47		0.30			
0.95		1.32		0.73			
5		8		6		2	
0.005		0.001		0.4			
64.3							
2.8							
5.6							
4							

0.206 ± 0.031 mM/l ($P < 0.005$). In the arterial blood a tendency towards an increase was noted 1 and 3 min after ending work ($0.025 < P < 0.05$).

The *XL factor* was 0.17 ± 0.20 mM/l at rest and increased significantly during work to 1.02 ± 0.18 mM/l ($P < 0.01$). One minute later a further significant rise had occurred and the XL factor then reached its maximum, i.e. 3.50 ± 0.95 mM/l ($P < 0.005$).

The *oxygen saturation* of the blood in the deep vein fell from a mean 59.2 ± 17.5 per cent at rest to 39.2 ± 15.2 per cent at the end of work ($0.01 < P < 0.025$). The oxygen saturation of the arterial blood remained unchanged during work.

The *heart rate* rose from a mean 65.7 ± 10.8 min at rest to 105.7 ± 7.3 at the end of work ($P < 0.001$). The return to the resting rate was exceedingly rapid, usually within 4–5 beats after the end of work.

Table IV Some data obtained in connection with forearm exercise of 5 sec duration

		Rest		End of work		15 sec after work	
		A	DV	A	DV	A	DV
Lactate	M	0.72	0.80	0.70	0.89	0.72	2.47
	SEM	0.04	0.05	0.05	0.07	0.07	0.26
	SD	0.14	0.18	0.17	0.26	0.23	0.89
	n	12	12	12	12	12	12
	P			0.5	0.2	0.95	0.001
Pyruvate mM/l	M	[illegible]	0.091			0.094	0.163
	SEM	0.005	0.006			0.006	0.020
	SD	0.016	0.017			0.017	0.057
	n	9	9			8	8
	P					0.2	0.005
XL mM/l	M	0.32				1.30	
	SEM	0.08				0.24	
	SD	0.25				0.68	
	n	9				8	
	P					0.005	
Heart rate	M	61.5		88.5		60.7	
	SEM	4.4		7.5		4.4	
	SD	10.8		18.4		10.7	
	n	6		6		6	
	P			0.005		0.3	

Symbols as in Table II

Work of 5 sec duration (Fig 6 Table IV)

In 8 cases forearm exercise was performed during only 5 sec The subjects were instructed to carry out as many contractions as possible during the work period The total work performed ranged from 2 1 to 2 5 kpm with a mean intensity of 27 8 ± 1 7 kpm/min

The *lactate concentration* in blood from the deep forearm vein was a mean 0 80 ± 0 18 mM/l at rest and rose to 0 89 ± 0 26 mM/l at the end of work this difference is not significant The concentration 15 sec after work was 2 20 ± 1 01 mM/l which is significantly higher than at rest ($P < 0\ 001$) A further rise occurred and 1 min after work the concentration was a mean 3 14 ± 0 90 mM/l After 30 min the concentration was essentially on the same level as at rest before work

The mean lactate concentration in the arterial blood was 0 72 ± 0 14 mM/l at rest No change had taken place at the end of work but 1 min later a tendency towards increase was registered ($0\ 025 < P < 0\ 05$)

The *pyruvate concentration* in deep venous blood increased from the resting level 0 091 ± 0 017 mM/l to 0 163 ± 0 057 mM/l 15 sec after work ($P < 0\ 005$) No changes were observed in arterial blood

1 min after work		3 min after work		5 min after work		15 min after work		30 min after work	
A	DV	A	DV	A	DV	A	DV	A	DV
0.97	3.14	0.84	2.88	0.92	2.43	0.72	1.23	0.74	1.02
0.07	0.28	0.10	0.19	0.09	0.11	0.07	0.11	0.07	0.06
0.26	0.96	0.34	0.65	0.23	0.29	0.24	0.37	0.23	0.18
12	12	[illegible]	12	7	7	11	[illegible]	10	10
0.05	0.001	0.15	0.001	0.05	0.001	0.8	0.01	0.9	0.05
0.083	0.130	0.085	0.157	0.083	0.140	0.080	0.110	0.077	0.093
0.006	0.008	0.006	0.009	0.007	0.008	0.005	0.007	0.004	0.005
0.016	0.024	0.017	0.076	0.018	0.021	0.015	0.021	0.012	0.013
9	9	[illegible]	9	7	7	9	9	8	8
0.4	0.005	0.5	0.001	0.5	0.005	0.6	0.02	0.1	0.7
1.61		1.11		0.90		0.21		0.15	
0.29		0.37		0.19		0.16		0.13	
0.88		1.10		0.49		0.49		0.36	
9		9		7		9		8	
0.001		0.005		0.01		0.2		0.3	

The *XL factor* had increased 15 sec after work ($P < 0.005$) and was still higher 45 sec later.

The *heart rate* was a mean 61.5 ± 10.8/min at rest and rose to 88.5 ± 18.4/min during work. After the end of work the rate usually returned to the resting level within the next 4—5 sec and in every case within 15 sec.

Discussion

The venous drainage of the forearm takes place not only through the subcutaneous venous system but also through three deep veins, i. e. the radial, ulnar and interosseus veins. Coles *et al.* (1958) have shown that the oxygen saturation of samples from the two first mentioned veins decreases when the circulation of the hand is occluded by inflating a cuff around the wrist. This has been interpreted as an indication that venous blood from the hand is drained to these veins at rest. It does not, however, seem to apply to the interosseous vein, which apparently lacks any communication with the vascular system of the hand.

These observations were confirmed by the present control studies in which indocyanine solution was infused into the radial artery. Dye could usually be

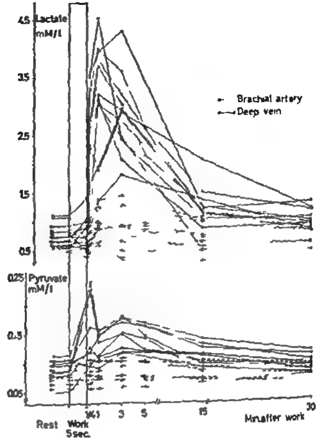

Fig 6 Lactate and pyruvate concentrations of arterial and deep venous blood of the forearm at rest and after work of 5 sec duration. Symbols in Fig 2

demonstrated in the deep forearm vein at rest but not during work of approximately 5 min duration The results of these experiments seem to show that the part of the circulation in the hand which derives from the radial artery does not in fact pass into the deep veins of the forearm in connection with rhythmic forearm work of high intensity This observation is further substantiated by the fact that occlusion of the cutaneous circulation of the hand and forearm by iontophoretic administration of adrenaline did not significantly alter the oxygen saturation of the deep forearm vein during work whereas it did so at rest (PERNOW and WAHREN unpublished) However despite this the question of to what extent samples from deep veins of the forearm taken during work contain non muscular blood cannot be regarded as finally settled, and it is intended to be the subject of further investigation

When evaluating the aerobic and anaerobic components of muscular metabolism it is not sufficient at least not when studying small groups of muscle to follow the lactate concentration of the arterial blood only which is apparent from the results on work of short duration Here the arterial lactate

remained practically unchanged during and after work whereas significant increase was recorded concurrently in the lactate concentration of blood from the deep forearm vein The same is true for submaximal work during long periods where a significant increase was obtained in lactate concentration of muscular blood without any change in arterial lactate (PERNOW and WAHREN, to be published) This observation of an unchanged lactate content of arterial blood in short work of high intensity is in agreement with earlier findings (CHRISTENSEN HEDMAN and SALTIN 1960) CHRISTENSEN *et al* concluded from their results that short, intense work could take place under completely aerobic conditions The present study shows however that a significant rise in lactate concentration occurs in the venous muscular blood, even during brief work of high intensity

When discussing the cause of this unchanged arterial lactate concentration in connection with work despite marked local formation there is reason to analyze the mechanisms by which lactate can be eliminated from the blood stream Both the liver and the heart actively utilize lactate and small quantities of it are excreted in the urine The lungs do not seem to take part in the metabolism of lactic acid (HOLMGREN 1958) Since lactic acid is freely diffusible it can be presumed that equilibrium is rapidly established between its concentration in the intra and extracellular fluid space (HUCKABEE 1956) Therefore at high arterial concentrations lactate will be taken up by *e g* resting muscle (BARR and HIMWICH 1923 EGGLETON and EVANS 1930 CARLSON and PERNOW 1959) Furthermore BUEDING and GOLDFARB (1943) found that infusion of lactic acid produced a rise in the arterial concentration of pyruvate which presumably implies that tissues containing lactic dehydrogenase convert lactate to pyruvate It is thus evident, that a normal arterial lactate concentration can be present even at a considerably increased local formation of lactate provided that this formation is either limited to a small group of muscles giving a large dilution of the lactate formed or does not exceed the rate at which it is eliminated from the blood stream

In work involving large groups of muscles unchanged high or rising arterial and venous lactate concentrations are often seen several minutes after ending work (ÅSTRAND 1960 CARLSON and PERNOW 1961) This can probably be attributed to a reduced lactate utilization rate after work and should not be taken as a criterion of the existence of anaerobic conditions at this time which was apparent from the simultaneous rapid reduction in XL values after the end of work This fact is further illustrated by the present observation that in connection with work of 2—7 min duration engaging small groups of muscles the lactate concentration of deep venous blood decreased immediately after the end of work although the lactate concentration was as high as in the previously mentioned study It is likely that the changes in lactate utilization rate during and after forearm exercise are smaller than in connection with exercise on a bicycle ergometer when larger groups of muscles are involved

The XL-values in this study have been calculated from AV-differences of lactate and pyruvate concentrations measured in blood samples obtained simultaneously. This is not strictly correct in connection with work of 2—7 min duration since there occurred changes in the arterial concentration. In order to be able to calculate correct AV differences one should arrange the blood sampling so that the venous sample is taken as much later than the arterial one as corresponds to one mean circulation time for the metabolite in question (ZIERLER 1961). In the case of the XL-values in connection with work of 2—7 min duration this would lead to slightly higher XL-values.

The oxygen saturation of deep venous blood decreased significantly during work of 2—7 min duration to a mean 24 per cent. There may be several reasons why no higher degree of oxygen utilization was obtained. As was shown by MONOD *et al.* (1961) the oxygen saturation of deep venous blood of the forearm decreased during work to a constant level which was independent of the work intensity. During work of 10—30 sec duration the oxygen saturation of deep venous blood fell to a mean 39 per cent. Judging by the pronounced rise in lactate which was observed concurrently the oxygen tension on the capillary level was not, however, sufficient for carbohydrate metabolism to take place under aerobic conditions. If the oxygen saturation of effluent venous blood is taken to indicate the oxygen tension on the capillary level this finding is in agreement with HUCKABEE's (1958) statement that even a moderate reduction of the oxygen tension causes a displacement of the equilibrium of the lactic dehydrogenase system towards lactate.

The changes in heart rate in connection with work of very short duration are strikingly rapid. It is likely that such a swiftly acting regulatory system operates by direct nervous action on the heart, possibly elicited from the hypothalamus as stated by SMITH, RUSHMER and LASHER (1960).

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Observations on Pigeons with Prethalamic Radiolesions in the Nervous Pathways from the Telencephalon

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Abstract

ÅKERMAN B, E. FABRICIUS, B LARSSON and L. STEEN. *Observations on pigeons with prethalamic radiolesions in the nervous pathways from the telencephalon.* Acta physiol. scand. 1962 56 286—298 — By local irradiation of the pigeon's brain with high energy protons, lesions were produced in the cerebral peduncles and the septal region at a level in front of the anterior commissure, without apparent damage to diencephalic structures. The behaviour of the animals following the procedure was observed. Spontaneous eating drinking and flying escape responses and all social behaviour disappeared during the first four days after irradiation, with a dose of 30 krad, in a group of pigeons in which all connections from the cerebral hemispheres were destroyed On the contrary swallowing blinking and pupillary reflexes as well as the muscular and visual control of standing walking and flying remained In another group of pigeons with small caudal parts of the peduncles intact similar behaviour changes were observed, but no pattern of behaviour disappeared until after five days or more. In a third group of pigeons in which only more anterior parts of the peduncles were transsected no behavioural changes were observed. The experiments demonstrate that delayed, circumscribed destruction of cerebral structures by means of ionizing radiation may be used to particular advantage in the study of the anatomical foundation of animal behavior

Beginning with ROLANDO (1809) a great number of physiologists interested in the role of the forebrain in the behaviour of birds have made ablation experiments, predominantly in pigeons The general appearance of a bird without

cerebral hemispheres seems to be well established it sits with ruffled feathers and displays no spontaneous activity However on some points there seems to be controversy over the results of such experiments In disagreement with observations made by FLOURENS (1824) BOUILLAUD (1830) and SCHRADER (1889) eating after complete isolation of the cerebral hemispheres was reported by MAGENDIE (1839) while SHAKLEE (1921) and van ESSEN (1932) were of the opinion that pecking at least persisted as a movement indicating eating intention ROLANDO (1809) BOUILLAUD (1830) and MUNK (1883) were of the opinion that pigeons with isolated cerebral hemispheres behave as if they are blind and unable to avoid obstacles while STEFANI (1881) and SCHRADER (1889) claimed that such birds can avoid obstacles when flying and that they are able to estimate distances of objects An extensive summary of the results of earlier investigations has been published by ten CATE (1936)

In spite of the work on the functional significance of the avian cerebral hemispheres as for instance by ROGERS (1922) and BEACH (1951) little is known about the role of the different striatal areas in the instinctive behaviour of the birds The experiments reported here were performed to investigate changes in behaviour following lesions in the pigeon s forebrain using a narrow beam of 185 MeV protons to produce well-defined radiolesions in the nervous tissue (*cf* LARSSON *et al* 1958) When this technique is used undesirable side-effects such as massive bleeding infection and surgical shock are minimized

An attempt was made to isolate completely the cerebral hemispheres of the pigeons by transsection of all their fibre connections with other parts of the central nervous system The behaviour of the birds was then observed at various times after irradiation and also with electrical stimulation of the intact diencephalic part of the forebrain The ethologic studies were followed by histologic examination of serial sections of the brains

Methods

Adult feral pigeons (*Columba livia*) of both sexes weighing 280—460 g at the time of irradiation were used The birds were kept in laboratory cages for about one week before operation

Irradiation technique

The radiosurgical operations were performed with the 230 cm synchrocyclotron at Uppsala The 185 MeV proton beam was controlled and the absorbed radiation doses were measured as described previously (LARSSON 1960 1961) Deep pentobarbital anesthesia was induced intraperitoneally about 30 min before irradiation The average dose of anesthetic was 3 9 mg pentobarbiton sodium dissolved in physiological saline immediately before use During irradiation the bird was placed on its back in a holder with conical ear plugs and a clip holding the upper part of the bill (Fig 1) The protons penetrated the skull transverse in the region slightly rostral to the anterior commissure, the cross section of the almost parallel beam being rectangular (2×5 mm²) The position of the pigeon in relation to the path of the beam was controlled by means of

Fig 1 Pigeon in position for irradiation The ionization chamber and the aperture defining the cross-section of the proton beam are seen in the upper right part of the photograph

Fig 2 Roentgenogram showing the typical position of the irradiated region in the pigeons of Group I The rectangular 5 × 2 mm cross-section of the proton beam has been visualized by autoradiography

roentgenograms (Fig 2) During irradiation the bird was observed by television and afterwards a second roentgen control of the position was made In no case were significant movements observed during irradiation and the position of the irradiated part of the skull in relation to the beam never differed by more than 1 mm from that before irradiation The radiation dose employed was 30 krad The dose rate was 1—3 krad/min

Technique of electrical stimulation

In order to study the effects of electrical stimulation of the intact brain structures near the caudal edge of the radiolesion 0 2 mm thick Pt Ir electrodes with 0 1—0 [illegible] mm uninsolated tips were sometimes implanted in this region as described by ÅKERMAN *et al* (1960) Unipolar stimulation was applied with a peak current of 0 05—1 0 mA and a tissue resistance of 7—10 kΩ The pulse duration was 3 msec and the frequency 50 *c/s* The DC component of the current was removed by a transformer at the output of the pulse generator

Observation technique

The pigeons were subjected to a series of daily routine tests in order to study their post irradiative behavioural changes In addition the eating was checked by weighing the birds daily and noting if any abnormal weight loss had occurred During the first days after irradiation the birds were given a certain number of peas every day and the remaining peas were counted next morning over longer periods daily weighing was a satisfactory method of checking the eating activity

Water was given in bowls filled to a certain level this was checked the next day It was also possible to learn if a bird had even dipped its bill in the water as this leaves a little powder on the water surface

To test the flying ability the pigeon was released in the laboratory the flight being regarded as spontaneous if the bird flew up from a stationary object without influence from the observer Furthermore the ability of the bird to avoid obstacles during flight the appearance of the flight and the ability of landing with precision were observed For birds which did not fly spontaneously the flying test was initiated by throwing them into the air

To investigate the presence of blinking reflexes rapid hand movements were made at a distance of 5—10 cm in front of the pigeon s eyes The region around the eyes also was touched to see if this resulted in a blinking reflex

The eyes of the pigeon were illuminated with an electric torch to ascertain whether the pupils contracted in a normal way The reaction to sound was tested by hand clapping and making peeping' noises In this test the pigeon was not able to see the observer

During the above mentioned tests the escape responses of the birds also were observed

The birds social behaviour was observed for shorter periods during which they were kept together with other pigeons

Histological procedure

The pigeons were generally allowed to live for periods of 1 to 5 weeks after irradiation depending on their condition (Table I) With the exception of pigeon no 4 which died of suffocation in connection with vomiting the birds were killed under anesthesia by exsanguination through an incision in the wall of the right ventricle Perfusion was performed at 150 cm H_2O with about 50 ml of a solution of 3 per cent gum acacia in physiological saline through 1 mm plastic cannulas inserted in the carotid arteries About 50 ml of the same perfusion liquid but with 10 per cent formalin were thereafter used to fix the brain by perfusion

The brains were removed and placed in 10 per cent neutral formal saline for about two weeks They were embedded in celloidin and cut transversally or sagittally in serial 20 μ sections The following staining methods were used Nissl for nuclei Gross-thionine for axons and nuclei and Loyez myelin sheath stain with neutral red contrast. Serial sections of normal brains were available for comparison

Results

Histological changes

No external morphological changes were observed The inner surface of the skin showed a slight yellowish discolouration at the site of irradiation The bone under the irradiated skin appeared slightly hyperemic In the brain the lesion was seen macroscopically as a band of grayish pink discolouration in the obvious path of the beam In some brains minute hemorrhages marked the border of the necrotized tissue

Fig 3 Transverse section of a pigeon's brain showing localized radiation damage in the obvious path of the beam, 9 days after irradiation with a dose of 30 krad Loyez × 3 4

Fig 4 Microscopic appearance of the edge of the radiolesion 9 days after irradiation with a dose of 30 krad The necrotic area (lower part of the figure) was surrounded by a narrow border zone with neurons in various stages of degeneration Nissl × 70

The microscopical appearance of the radiolesion was similar to that observed earlier in rabbits irradiated under similar conditions (LARSSON *et al* 1958 1959, REXED *et al* 1960) The rectangular cross section of the necrotic lesion corresponded well to that of the beam (Fig 3) In the fixed material the width of the lesion was about 2 mm All neural tissue components within the central part of the lesion were destroyed The necrotic part of the lesion was surrounded by an irregular border zone 0 5—1 mm wide with neurons in varying stages of degeneration (Fig 4) Outside this zone the nervous tissue appeared normal except for the secondary Wallerian degeneration The estimates given below of the extension of the lesions are approximate the boundary of the lesion being somewhere in the middle of the border zone where the tissue appeared neither entirely necrotic nor fully normal

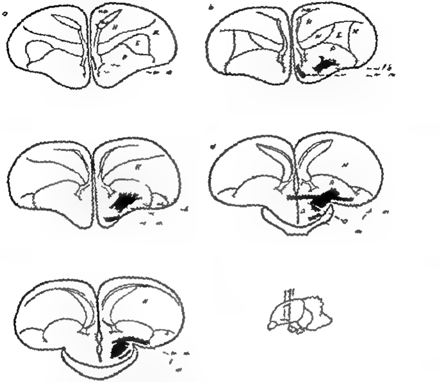

Fig 5 Schematic figures from transverse sections of the brain of a pigeon with complete nervous isolation of the telencephalon The lesion is shown as a shaded area.
Abbreviations A = Archistriatum a. c = anterior commissure D = Diencephalon E. = Ektostriatum H a = Hyperstriatum accessorium H d. = Hyperstriatum dorsale H v = Hyperstriatum ventrale l f d = lateral forebrain bundle N = Neostriatum P = Paleostriatum S = Septal area tr o m = tractus occipito-mesencephalicus tr s m. = tractus septo-mesencephalicus

Extension of the lesions

An ideal lesion disconnecting completely the telencephalon from other parts of the brain would be located in the paleostriatum the ventromedial forebrain wall and the septal area In some attempts to produce prethalamic lesions of this kind all connections were successfully destroyed (group I) while in others the most caudal parts of the peduncles were partially spared (group II) In a few birds only the more anterior parts of the peduncles were destroyed while a great deal of the connections were left intact (group III) Six pigeons with lesions which could not be ascribed to any of these groups were excluded from the experimental material considered in this paper

Group I Pigeons with complete nervous isolation of the telencephalon In 6 pigeons all connections between the telencephalon and the lower parts of the brain were cut The edge of the well demarcated lesion was seen in the anterio-

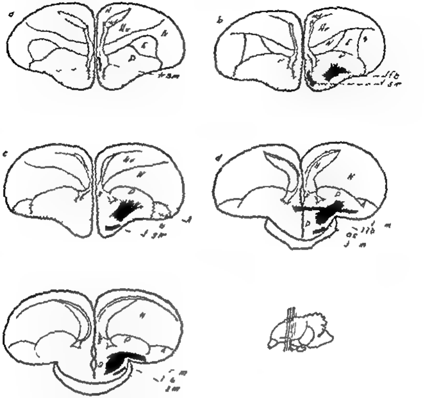

Fig 6 Schematic figures from transverse sections of the brain of a pigeon with incomplete nervous isolation of the telencephalon The cerebral peduncles posterior to the anterior commissure are intact For abbreviations see fig 5

ventral paleostriatum Caudally the paleostriatum, the nuclei of the ventromedial forebrain wall and the septal area were destroyed There were no signs of damage either to the thalamus or hypothalamus (Fig 5)

Group II Pigeons with incomplete nervous isolation of the telencephalon The cerebral peduncles posterior to the anterior commissur were intact In 12 pigeons the lesions were located more rostrally than in the pigeons of the previous group The paleostriatum in front of the anterior commissure, the nuclei of the ventromedial forebrain wall and the septal area at this level were invariably necrotic in this group The small part of the cerebral peduncles posterior to the anterior commissure and the paleostriatum at this level were however, intact Thus all the connections between the telencephalon and the lower parts of the brain were intersected except for a bundle of fibres belonging to the tractus occipitomesencephalicus The diencephalon was undamaged (Fig 6)

Group III Pigeons with incomplete nervous isolation of the telencephalon The cerebral peduncles posterior to the anterior commissure and parts of the cerebral peduncles and the

Table I The development of behaviour changes in the irradiated pigeons

Group	Pigeon no	Spontaneous eating and drinking disappeared	Escape responses disappeared	Spontaneous flight disappeared	Landing precision weakened	Ability of avoiding obstacles when flying disappeared	Blinking reflexes disappeared	Pupillar reflexes disappeared	Reaction to sound disappeared	Killed or died
I	1	1	1	1	—	—	—	—		20
	4	1	1	1	—	?	—	—	?	3
	9	1	1	1	3	—	—	—	?	6
	11	1	1	1	—	—	—	—	?	5
	13	1	1	4	4	—	—	—	1	9
	21	1	1	1	—	—	—	—	1	14
II	2	8	9	9	—	—	—	—	—	14
	3	7	[1]7	11	—	—	?	?	7	13
	5	8	9	—	—	—	—	—	—	10
	6	7	7	7	—	—	—	—	—	10
	7	7	7	6	—	—	—	—	—	7
	8	6	6	6	—	—	—	—	—	7
	14	10	15	14	—	—	—	—	14	30
	15	7	26	25	—	—	—	—	—	37
	17	5	16	17	—	—	—	—	14	23
	18	7	9	21	—	—	—	—	—	32
	[illegible]	6	7	—	—	—	—	—	—	14
	24	5	14	10	—	—	—	—	—	14
III	12	—	[2]—	—	—	—	—	—	—	20
	16	26	—	26	—	—	—	—	—	37
	19	—	—	—	—	—	—	—	—	40
	20	21	—	—	—	—	—	—	—	23

[1] Escape responses did not disappear entirely but were very weakened
[2] Very strong escape responses.

— signifies that the behaviour in question did not weaken or disappear

The figures indicate time after irradiation in days.

septal area in front of the anterior commissure were intact In four pigeons the lesions were located 0 5—1 0 mm more rostrally than in the pigeons of group I As a consequence some tissue belonging to the paleostriatum and the septal area in front of the anterior commissure was also intact

Ethologic observations

The following account gives a description of the typical behaviour of the pigeons belonging to the three different groups The ethologic observations made in each bird have been condensed into Table I which should be consulted for further details

Group I The behaviour of birds with lesions causing complete nervous isolation of the telencephalon Spontaneous eating and drinking had disappeared already during the first day after irradiation and these birds lost weight successively during the following days The swallowing reflex seemed to be normal, since peas placed deeply in the bill were easily swallowed The swallowing reflex was similarly released when the bill was kept under water

The escape responses disappeared entirely during the first day after irradiation and the birds never flew spontaneously after 1—4 days If these birds were thrown into the air after this time they were still able to fly normally, avoid obstacles and land with full precision They also avoided obstacles when walking was induced No abnormalities were observed in the blinking and pupillar reflexes

Two of these birds (no 13 and 21) did not react to sound on the first day after irradiation The observations in the other four animals of this group were difficult to interpret and it is uncertain whether or not these birds could hear

After irradiation neither calling nor any of the postures or movements belonging to the behaviour patterns of fighting or courting were ever observed Balancing movements persisted and the birds were able to stand in a normal position When not disturbed the members of this group already by the first day after irradiation, sat motionless with eyes closed and feathers ruffled

In four of these completely radio detelencephalated birds electrodes were implanted into the anterior thalamus and hypothalamus proximally to the areas destroyed by the proton beam With stimulation of this undamaged part of the brain all the pigeons showed increased activation Both ipsiversive and contraversive deviation of the head and trunk, turning into circling movements, were induced as well as depression and raising of the head The pigeons could also in this way be made to walk and spread out their wings as well as to close their eyes and contract their pupils In some pigeons stimulation caused erection of the feathers of the neck. Defecation and preening were also observed

Sometimes the movements of the head and the walking of the stimulated birds gave the impression of "attention and searching but the birds remained unaffected by external stimuli such as food and water other pigeons or pigeon dummies

Group II The behaviour of birds with incomplete nervous isolation of the telencephalon The cerebral peduncles posterior to the anterior commissure intact The birds belonging to this group lost their ability of spontaneous eating and drinking 5 to 10 days after irradiation Like the pigeons of group I they retained their swallowing reflex

The escape responses disappeared 6 to 26 days after irradiation In one bird (no 18) these responses never disappeared completely within the time of observation but became very weak.

Six to 25 days after irradiation these pigeons with two exceptions, ceased to fly spontaneously but retained the ability to fly normally when thrown into the air They were also able to avoid obstacles and finish the flight by landing with precision

Like the pigeons of group I they exhibited normal blinking and pupillar reflexes There was also an obvious reaction to sound except for three birds (no 3 14 17) in which such a reaction disappeared 7 to 14 days after irradiation.

When the preoptic area the anterior thalamus and hypothalamus were stimulated in six of the pigeons in this group the birds were activated similar responses being elicited as with stimulation in Group I

Group III The behaviour of birds with incomplete nervous isolation of the telencephalon The cerebral peduncles posterior to the anterior commissure and parts of the cerebral peduncles and the septal area in front of the anterior commissure were intact None of these birds lost the ability of spontaneous eating and drinking until at least 20 days after irradiation Two of the birds (no 12 and 19) retained these abilities until they were killed

The escape responses in these pigeons never disappeared In two (no 12 and 29) there were even extraordinarily strong escape responses although they had been quite normal in this respect before irradiation

One pigeon (no 16) ceased to fly spontaneously after 26 days With this exception the birds of this group were normal with respect to flight avoiding obstacles and landing precision Their blinking reflexes pupillar reflexes and reaction to sound were also normal

These birds also seemed to show normal social behaviour Thus they were observed calling and bowing at the sight of other pigeons

Discussion

Narrow beams of ionizing particle radiation were successfully introduced as operative instruments in experimental physiology by TOBIAS *et al* (1954 1955) in their attempts to affect the function of the pituitary In experiments reported by LARSSON *et al* (1958) and LEKSELL *et al* (1960) it was demonstrated that such beams can also be used to advantage for the production of well circumscribed delayed lesions in the central nervous system suitable for the study of deep-lying cerebral structures The experiments described here show the particular usefulness of this technique in investigations concerning the anatomical basis for the patterns of animal behaviour

As landing precision and the ability of avoiding obstacles during flight and in walking require the ability to estimate distances and to discern objects the

present results confirm the findings of RENZI (1864), STEFANI (1881) SCHRADER (1889), NOLL (1915) and VISSER (1932), that pigeons possess a certain capacity of sight without the co operation of the telencephalon The opposite view was held by FLOURENS (1824), *BOUILLAUD (1830)* and MUNK (1883) The fact that complete isolation of the telencephalon by no means deprives the pigeon of its visual percipience is of great significance for considerations of other functions of the pigeon s forebrain

The observations indicate that pigeons are not able to eat and drink spontaneously without participation of the telencephalon Neither are they able to perform escape responses as observed by SCHRADER (1889) NOLL (1915) and SHAKLEE (1928) or spontaneous flight as observed by BISCHOFF (1863) and VOIT (1868)

Incomplete isolation of the telencephalon, with small caudal parts of the cerebral peduncles spared, caused the disappearance of the same behaviour patterns as after complete transsection of the nervous pathways from the telencephalon although the effect of the irradiation was apparent at a later stage after the irradiation When only frontal parts of the peduncles were intersected no disturbances in behaviour were observed within the first three weeks after irradiation On the contrary, the escape responses in two birds with such lesions even became exaggerated during the postoperative period an interesting fact which cannot be explained as yet

All pigeons studied possessed normal swallowing blinking and pupillary reflexes and they were able to perform the muscular control necessary for maintaining a stable normal posture and for performing normal flight and landing

The observations of reactions to sound are difficult to interpret Even pigeons apparently deprived of all connections with the telencephalon were frequency seen to open their eyes in apparent response to noises It seems plausible in view of the anatomy of the pigeon s brain to postulate that *these birds* possessed audial perception although it was difficult to induce any response even by strong stimuli

Stimulation of the basal forebrain of normal pigeons by means of permanently implanted electrodes induced complete instinctive actions such as eating drinking and bathing as well as some behavioural patterns belonging to the named *social behavioural pattern of the bird* (ÅKERMAN *et al* 1960) Stimulation of the same region in birds with the telencephalon isolated elicited no such responses The birds were activated but the consummatory act was *never* performed

The results of the experiments indicate that the telencephalon of the bird is concerned with the recognition of stimuli related to some categories of instinctive behaviour, such as feeding drinking escape behaviour and *social* behaviour and with the orientation of the bird and its movements in relation to these stimuli The subtelencephalic structures seem to be able to control *not* only all

important reflexes including the basic motor patterns of walking and flying but also the basic visual control of the behaviour associated with locomotion

The great importance of the basal ganglia for the behaviour of the pigeon has been stressed by for instance ROGERS (1922) who did not observe any changes in behaviour after decortication A more detailed analysis of the behaviour changes induced by radiolesions in the striatal areas of the pigeon is in progress It is hoped that such studies can form a basis for comparison of behavioural changes of pigeons with lesions in the basal ganglia and such changes in decorticate mammals of varying degree of cerebral development

To the authors knowledge a selective isolation of the telencephalon has not been made in any adult mammal The production of a suprathalamic preparation , as described in this paper can in fact be performed much easier in birds with their extended cerebral peduncles than in mammals

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Acta physiol scand 1962 56 299—305

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Breath-by-Breath Sampling of Alveolar (End-Tidal) Gas

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Abstract

Brismar, J C M Hesser and G Matell. *Breath by-breath sampling of alveolar (end tidal) gas* Acta physiol scand 1962 56 299—305 —For studies of the rate of change of alveolar gas composition in transitional states a breath by breath end tidal gas sampler is proposed which combines great simplicity in design with a much reduced mixing of successive samples The validity of the method is discussed and illustrated by breath by breath comparisons of end tidal and arterial P_{CO_2} the latter being followed by continuous recording of the arterial pH It is shown that in or near the resting conditions the two parameters closely follow each other even when exhibiting rapid and great fluctuations and that the P_{CO_2} of the samples approximates the functional mean alveolar P_{CO_2}

There is at present no method available for the truly continuous sampling of alveolar gas consequently the best that can be achieved by the use of rapid and automatic gas analyzers is the consecutive analysis of a discontinuous series of representative gas samples However with the increasing interest devoted to the dynamics of pulmonary gas exchange it is not surprising to find a definite need for a convenient method that does permit delivery of such representative samples under varying conditions not only during steady state or slowly changing alveolar gas composition but also during short term shifts from one level to another A new end tidal gas sampler is proposed which combines great simplicity of design with a much reduced mixing of successive samples and which can thus conveniently be used with rapid gas analyzers to study breath by breath changes in end tidal gas composition The theoretical background of its principle of operation is discussed and illustrated by breath by breath comparisons of the P_{CO_2} of sampled gas with that of the arterial blood

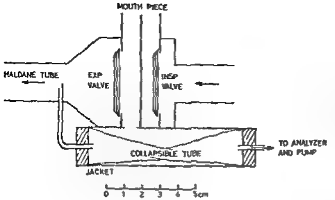

Fig 1 Diagram of end tidal gas sampler unit including low dead space breathing valve assembly Only *inside* dimensions of valve assembly jacket and connecting tubes are indicated

Requirements and Principle of Sampling

A method for alveolar gas sampling should preferably fulfill the following requirements

a) It should permit an automatic breath by breath sampling of gas the composition of which agrees with that of the functional mean alveolar air even with rapidly changing gas tensions

b) It should not interfere with respiration *e g* by significantly increasing the dead space or by requiring a greater respiratory resistance for its proper function than is created by a low resistance breathing valve system. When used in human experiments it should be noiseless in order not to disturb the subject

c) No supervision or adjustment should be needed under changing experimental conditions

Fig 1 shows the dimensions and constructional details of the present sampler On a low dead space (about 10 ml) breathing valve assembly (v DÖBELN 1949) a lucite tube is mounted on the opposite side to the mouthpiece A large hole connects the tube with the space proximal to the outlet valve A thin walled rubber tube (a condom with the blind end cut off) is placed inside the lucite tube and kept in position by means of two rubber stoppers connections being made to a HALDANE expiratory tube and to the analyzers The sampler in Fig 1 is dimensioned for human subjects and for the continuous delivering of about 100 ml/min of end tidal gas

For satisfactory function the mounting of the collapsible tube should be done as follows The wall distal to the mouthpiece should be a little more stretched than the quite relaxed wall facing the mouthpiece Furthermore when inserting the rubber stoppers they should be counter rotated about 30° to obtain a slight twisting of the collapsible tube, so that the latter tends to assume the shape of an hour glass

When gas is continuously drawn from the collapsible tube through the gas analyzers (at a constant rate of about 100 ml/min, using a small suction pump) the following events take place During inspiration the proximal end of the

expiratory tube contains end tidal gas from the preceding exhalation Part of this gas will be drawn into the collapsible tube as it expands owing to the negative pressure in the lucite housing during inspiration The positive pressure created during the ensuing expiration will compress the tube and thus push back part of its gas content to the expiratory tube thereby preventing sampling of gas (dead space air) from the latter during the first phase of expiration As the expiration and the compression of the collapsible tube proceed the middle portion of the tube will eventually close, leaving a small conical gas pocket on each side The volume of the pocket facing the analyzers is usually sufficiently large to permit continuous gas supply to the analyzers even during the last phase of the expiration This sequence of events is repeated during each respiratory cycle and thus a breath by breath sampling of end tidal gas is achieved Normally then the gas analyzed during a given respiratory cycle will be the end tidal portion of the preceding exhalation

In the event of a long expiratory pause the middle portion of the collapsible tube will again open so that sampling of end tidal gas now is done directly from the expiratory tube Thus during slow respiration the end tidal gas analyzed during a given respiratory cycle originates first from the preceding exhalation and later, from that in progress

Discussion

Since HENDERSON and HAGGARD in 1925 proposed the first automatic method for selective sampling of end tidal (end-expiratory) gas a number of improved devices have been produced In recent years mainly two principles have been used *viz* 1) that originally described by HESSER (1949) and subsequently employed also by PALMER (1958) and by LAMBERTSEN and BENJAMIN (1959) and 2) that of RAHN *et al* (1946 1949) The former methods permit a sample of the last portion of each exhalation to be withdrawn from any point within the upper respiratory passages or from beyond the exhaling valve of a breathing system The intermittent drawing of gas is achieved by means of a pressure switch relay solenoid system governed by the pressure pattern of the respiratory cycle When used in combination with automatic and rapid gas analysis this technique permits a study of breath by breath changes in end tidal gas composition even at rather small tidal volumes and/or with rapid changes in gas tensions

With the device described by RAHN *et al* (1946 1949) samples of end tidal gas are drawn from the proximal end of a smooth bore tubing which is connected to the expiratory side of a breathing valve system The more recent version is entirely mechanical in principle the work of sampling being provided by the respiratory pressure fluctuations in the breathing valve system The device consists of a small thin walled rubber balloon inside a glass housing the balloon being connected with the end tidal sample line and the glass housing

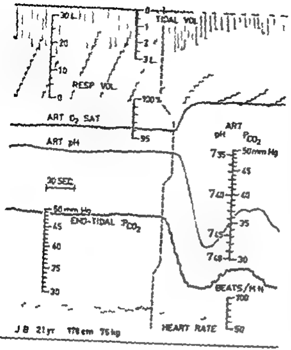

Fig 2 Rate of change of end tidal P_{CO} and arterial pH (P_{CO}) when at the vertical dashed line 100 O is suddenly administered to a resting subject after breathing a gas mixture of 6 3 CO_2, 15 9 O_2 and 77 8 % N_2 for 8 min. Gas from the sampler was drawn through a Beckman Spinco CO_2 infrared analyzer (model LB-1) at a constant rate of 100 ml/min The calibration scale for arterial P_{CO_2} was calculated from the HENDERSON HASSELBALCH equation, omitting the slight changes in the blood bicarbonate content caused by changes in O saturation. Vertical dashed line also indicates the time lags after which pulmonary events are reflected in the tracings (iso-time line)

with the breathing valve system During inspiration the negative pressure in the latter system will create an inflation of the balloon and end tidal gas from the fore going exhalation is drawn into the balloon During the ensuing expiration the positive pressure in the glass housing causes the balloon to collapse slowly and part of its gas content is pushed back into the expiratory tube In this way the first portion of the expired gas (dead space air) is prevented from being drawn into the analyzing apparatus The extreme simplicity of design has lead to a wide-spread use of this ingenious device However because the balloon does not collapse completely during each single expiration any single sample will represent a mixture of samples drawn over the preceding 1—3 min period (LUNDOREN 1958) This mixing delay is of little or no importance under steady state or slowly changing conditions but will become significant when rapid and large changes in the end tidal gas composition take place

By contrast the present device when employed with rapid and continuous gas analyzers permits a study of breath by breath changes in end tidal gas composition even when such changes are large and rapid This is illustrated in Fig 2 which shows the rate of change of end tidal P_{CO} in a healthy subject sitting upright when the gas inhaled is suddenly changed from a mixture of 6 3 % CO 15 9 % O_2 and 77 8 % N_2 to 100 % oxygen The time pattern of the end tidal P_{CO} is shown to follow closely that of the simultaneously recorded arterial pH (P_{CO}) indicating a proper performance of the sampler That in resting conditions the P_{CO} of the end tidal samples provides a close approxima

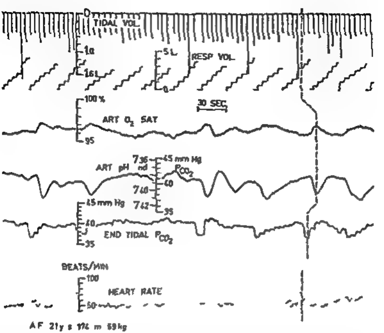

Fig 3 Synchronous fluctuations of end tidal P_{CO_2} and arterial pH (P_{CO_2}) and O_2 saturation in a resting supine subject breathing air Note their amplitudes and time-courses secondary to spontaneous irregularities in the respiratory pattern Recording technique and calibration as in Fig 2 Vertical dashed line = iso-time line.

tion to the arterial and hence to the mean alveolar P_{CO_2} is illustrated in Fig 3, which shows the spontaneous and synchronous fluctuations of end tidal P_{CO_2} and arterial pH (P_{CO_2}) in a healthy subject breathing air in the supine position In Figs 2 and 3 arterial pH inspired tidal and respiratory minute volumes (BTPS) arterial O_2 saturation and heart rate were continuously recorded using techniques described elsewhere (Bjurstedt *et al* 1962)

Any form of end tidal sampling of alveolar gas requires that the gas interposed between the alveolar space and the point of withdrawal of the samples is effectively washed out The expired volume required to wash out the dead space has been referred to as the kinetic dead space (Lilly 1946) and found to be at least twice as big as the anatomical dead space (Fowler 1948, Nunn and Pincock 1957) When sampling from a point just beyond the expiratory valve of a low dead space breathing system as done with the present technique the tidal volume should therefore in normal resting conditions exceed 400—500 ml in order to get alveolar gas That end tidal samples as obtained with the present method accurately represent the mean alveolar gas when the tidal volume is larger than about 450 ml (BTPS) has recently been shown in this laboratory (Bjurstedt *et al* 1962) In 5 healthy resting subjects the tidal volume end tidal P_{CO_2} and arterial pH were recorded continuously and

simultaneously while the subjects were raised from the supine to the standing position When lying down the tidal volume averaged 553 ml (range 439—673), the end tidal P_{CO_2} 38 7 mm Hg and the calculated arterial P_{CO_2} 39 5 mm Hg The arterial — end tidal P_{CO_2} difference thus averaged 0 8 mm Hg, which agrees with the theoretically calculated value for the arterial — mean alveolar P_{CO_2} gradient (*cf* BRISCOE 1959)

Without an adequate dead space washout, there will be a contamination of the alveolar gas sample with gas from the dead space of the subject and breathing apparatus, which will result in the creation of a significant difference between the gas composition of the end tidal samples and that of the alveolar air However if caused by occasional appearance of less than normal tidal volumes such hypoventilation artefacts are easily identified when the breath by breath sampling method is combined with continuous recording of the end tidal P_{CO_2} This would not be possible with such end tidal gas samplers (RAHN *et al* 1946, 1949) in which a significant mixing of samples from successive exhalations occurs

Because of the small dimensions of the present sampler the gas volume outside the collapsible tube in the lucite housing is changed by only about 5 ml during each respiratory cycle which means that the functional dead space of the breathing system used is also increased by about 5 ml The resulting increase in tidal and minute volumes will not show up in gas volume measurements since the sampler is not placed in series with the respiratory passages Therefore if the end tidal gas that is continuously withdrawn (100 ml/min) by the pump is repassed to some distant point in the expiratory tube measurements of tidal and minute volumes as well as of mixed expired gas composition will not differ significantly from the values obtained in case no sampler is used If on the other hand the end tidal gas is not repassed to the expiratory tube the measured expiratory (but not the inspiratory) minute volume will become 100 ml less A minimal change in gas composition of the mixed expired air will also take place in this case (*cf* NYE and RAHN 1955)

The straight and large communication between the breathing valve and the lucite housing secures a direct action on the collapsible tube of the respiratory pressure fluctuations which has the advantage that the sampler works properly also with breathing valves of very low resistance

Apart from the need of occasional replacement of the collapsible tube, which is accomplished quite easily no maintenance of the sampler is required Once the collapsible tube has been inserted correctly the device needs no adjustment or supervision no matter whether *e g* the respiratory frequency changes rapidly The reliability of the sampler has been proved also under rather adverse conditions such as under the influence of increased gravity (5 *g*) in the human centrifuge or at increased atmospheric pressures in a recompression chamber Since the device contains no mobile parts other than the collapsible rubber tube, the work of sampling is noiseless

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The Rate of Disappearance of Vasoconstrictor Responses to Sympathetic Chain Stimulation after Reserpine Treatment

By

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Abstract

ROSELL, S and G SEDVALL *The rate of disappearance of vasoconstrictor responses to sympathetic chain stimulation after reserpine treatment* Acta physiol scand 1962 *56* 306—314 — Vasoconstrictor responses in the vascular bed of skeletal muscles of cats were studied after administration of reserpine (5 mg/kg b w i v) The vasoconstrictor responses were produced by electrical stimulation of the sympathetic chain Sympathetic decentralization largely precluded the disappearance of the responses after reserpine treatment With increasing total number of stimuli applied to the vasoconstrictor nerves the magnitude of the vasoconstrictor responses gradually decreased After delivery of about 10 000 stimuli the vasoconstrictor responses had almost completely disappeared It is suggested that the normal impulse discharge is an important factor in transmitter substance depletion at the nerve endings following reserpinization The depletion does not seem to be followed by repletion

Reserpine depresses the level of catechol amines in different organs (BERTLER CARLSSON and ROSENGREN 1956) In the adrenal medulla this depletion can be reduced to some extent by denervation HOLZBAUER and VOGT (1956) found that denervation completely protected the adrenal medulla of cats from catechol amine depletion after small doses of reserpine (0 4 mg/kg) MUSCHOLL and VOGT (1958) extended these studies and showed that denervation produces only a partial protection after larger doses of reserpine (2 5 mg/kg or more) KRONEBERG and SCHUMANN (1957) demonstrated that denervation reduces but does not prevent the loss of catechol amines from rabbit adrenals It is conceivable therefore that at least for the adrenal medulla the central nervous

system is in some way involved in the mechanism by which reserpine exerts its depletory effect (BRODIE *et al* 1957) This suggestion is supported by the finding that ganglion blocking agents antagonize the depletory action of reserpine on adrenal glands in rats (KARKI, PAASONEN and VANHAKARTANO 1959, MIRKIN 1961) MIRKIN suggested that the catechol amine depletion in peripheral organs results from a dual action of reserpine The degree of depletion would thus depend upon the effect of reserpine both on the central nervous system and on the peripheral effector unit After having recorded the action potentials in small strands of the preganglionic cervical sympathetic nerves in cats IGGO and VOGT (1960) concluded that reserpine (1 mg/kg for several days) did not fundamentally alter the preganglionic discharge The discharge it is true was more continuous and possibly more frequent after reserpine but these findings do not explain the mechanism by which the central nervous system affects the depletory action of reserpine

This investigation was conducted with the aim of elucidating the quantitative influence of the nervous outflow on the action of reserpine on adrenergic nerves The responses to electrical excitation of adrenergic vasoconstrictor nerves supplying skeletal muscle vessels were registered under various conditions These nerves are generally thought to have noradrenaline as transmitter substance The change in tissue catechol amine content after reserpine was not measured in this investigation as the normal amine level in skeletal muscle is at the borderline of the sensitivity of available methods

Methods

The experiments were performed on cats weighing 2 0 to 4 2 kg under urethane (480—1240 mg/kg) anesthesia The trachea was cannulated Arterial pressure was recorded from a carotid by mercury manometer or Statham pressure transducer (P 23 AA) One or both abdominal sympathetic chains were isolated via the anterior approach Transection was done at the level of L_4—L_4 Stimulation was produced by a bipolar silver electrode applied to the distal part of the transected sympathetic chain. When the two chains were simultaneously stimulated they were brought into contact with the same electrode Supramaximal stimulation voltage and impulse duration was used throughout The stimulation frequency was varied The stimulator was a Grass Model S 4

Muscle blood flow was measured in a hind limb by cannulating the femoral artery and directing the blood through a silicone filled drop chamber The number of drops was registered by means of a photocell unit which operated an ordinate writer (LINDGREN 1958) The blood re-entered the hind limb via the cannulated distal stump of the same artery For selective measurement of the muscle blood flow the hind limb was skinned, and a tight ligature around the ankle isolated the paw from the circulation As a rule the blood flow was measured in both hind limbs Intra arterial injections were given through a side arm of the arterial drop chamber To prevent clotting heparin (25 mg/kg) was given i v Dextran was administered i v as required to compensate for blood loss

Rectal temperature was maintained at 36—37 C by means of a heating lamp The vascular resistance was calculated as the ratio of arterial pressure to flow

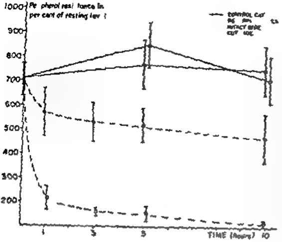

Fig 1 Peripheral resistance in per cent of resting level during stimulation of sympathetic chain at different times after reserpine administration Resting level = 100 % Reserpine was administered (5 mg/kg i v) at zero time Control animals were not treated with reserpine In all animals 0 5 mg/kg atropine was given i v in order to block the effects of vasodilator nerves Intact side signifies that the sympathetic chain was left intact until just before the stimulation Cut side indicates transection of the sympathetic chain prior to reserpine administration.

The sympathetic outflow was stimulated for 30 sec with supramaximal voltage and impulse duration. The stimulation frequency was 10/sec

The vertical bars indicate plus or minus the standard error of the mean Each point represents the mean value of 5 experiments

Substances used

Reserpine (Serpedin® Pharmacia Serpasil Ciba) 2 5 %
Noradrenaline (Noradrenaline Conc Byk Gulden L-noradrenalin bitartr)
Dextran (Macrodex Pharmacia)
Heparin (Heparin Vitrum) 5 %
Atropine (Atropini sulfas Sw Ph XI)
Noradrenaline is expressed as base in data concerning dosage given

Results

In one series of experiments the magnitude of vasoconstrictor responses to sympathetic chain stimulation was measured at various intervals after i v administration of reserpine In order to block the effects of cholinergic vasodilator nerves atropine (0 5 mg/kg i v) was administered Both abdominal sympathetic chains were dissected free and one of them was transected at the level of L_4—L_5 Reserpine (5 mg/kg) was then administered i v After varying intervals (1 3 5 or 10 h) the other sympathetic chain was transected Both hind legs were prepared for blood flow measurements (see Methods) and the recording of blood flow was started The peripheral ends of both sympathetic chains were then excited electrically with supramaximal stimuli The experimental results are seen in Fig 1 In control animals which had not received reserpine the responses to supramaximal stimuli remained relatively constant for several hours whether or not the autonomic nervous supply to the skeletal muscles had been left intact In cats treated with reserpine, on the other hand there was a clearcut quantitative difference between the responses in the two hind legs It was thus apparent that the presence of an intact sympathetic nervous supply during the interval between administration of reserpine and

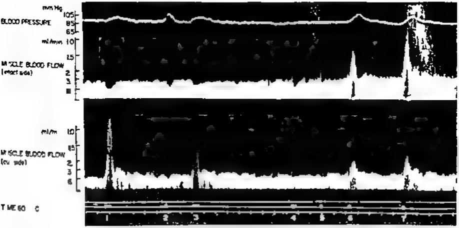

Fig 2 Cat 2 9 kg Urethane 830 mg/kg Reserpine 5 mg/kg i v 10 hours prior to the experiment Responses in skeletal muscle vessels to sympathetic chain stimulation and to intra arterial injection of noradrenaline "Intact side" signifies that the sympathetic chain was left intact until just before the stimulation. "Cut side" indicates transection of the sympathetic chain prior to reserpine administration Injections were given into the right and left femoral arteries simultaneously

1 Stimulation 5 V 10 imp/sec 2 0 5 mg/kg atropine i v 3 Stimulation 5 V 10 imp/sec 4 0 2 ml saline i a bilaterally 5 0 2 ml saline i a bilaterally 6 0 5 µg noradrenaline i a bilaterally 7 1 0 µg noradrenaline i a bilaterally

stimulation was an influential factor in the subsequent response On the "intact" side the responses to a supramaximal stimulus almost disappeared within 2 hours whereas on the denervated side pronounced vasoconstrictor responses persisted for at least 10 hours

The difference in vasoconstrictor responses between the two sides after reserpine administration could be due in part to a change in sensitivity to the transmitter substance Both sympathetic denervation and decentralization are known to produce supersensitivity to adrenaline and noradrenaline It has been reported moreover that supersensitivity to noradrenaline develops after reserpine administration (FLEMING and TRENDELENBURG 1961) The persistance of pronounced vasoconstrictor responses long after transection of the abdominal sympathetic chain might be explained by an increased sensitivity to the released transmitter substance on the denervated side This possibility was tested by injecting noradrenaline intra arterially Fig 2 illustrates such an experiment Five mg/kg reserpine was administered i v 10 hours before the start of the experiment Prior to reserpine administration the right sympathetic chain was transected at the level of L_4—L_5 whereas the other sympathetic chain was left intact until just before the electrical excitation Fig 2 1 illustrates the vasomotor responses to stimulation of the peripheral ends of both sympathetic chains On the "intact" side there was an increase in blood flow whereas the stimulation caused a decrease in blood flow on the "transected" side Atropine (2) reduced

the response on the "intact" side (3), indicating that cholinergic vasodilator nerves had been activated. The remaining increase in blood flow on the "intact" side after atropine probably merely results from the rise in blood pressure following the vasoconstriction in the other leg. At 6 and 7 noradrenaline (0.5 μg and 1.0 μg respectively) was injected simultaneously into the left and right femoral arteries. The vasoconstrictor responses in the right hind limb were no more pronounced than those in the left. Thus there was no indication of an increased sensitivity to noradrenaline as a result of sympathetic decentralization 10 hours prior to the stimulation.

It is reasonable to suggest that the rapid decline of vasoconstrictor responses on the "intact" side was due to a continuous release of the transmitter substance from the nerve terminals by nerve impulses, whereas on the "transected" side propagation of nerve impulses from the central nervous system was not possible. It could be argued, however, that the differing vasoconstrictor effects might be due to differing resting blood flows in the two limbs, FOLKOW (1962) having shown that a reduction in blood flow considerably affects the responses to sympathetic chain stimulation. In our series of experiments, however, the blood flow was generally of about the same order in the two hind limbs. Thus the differing responses in the two limbs could not have been attributable to a disparity in resting blood flows.

A series of experiments was then carried out with the aim of testing the hypothesis that the normal impulse discharge is instrumental in the depletion of transmitter substance after reserpine. It was sought to ascertain whether a ..ationship exists between the total number of stimuli delivered to the sympa... outflow and the decrease in vasoconstrictor responses. Both sympathetic ..hains were transected at the level of L_4—L_5. To preclude effects of vasodilator nerve excitation, atropine (0.5 mg/kg) was given. Supramaximal electrical stimuli with a frequency of 10 imp/sec were then applied to the peripheral ends for 30 sec. This type of stimulus was called the test stimulus. The vasoconstrictor response thus obtained was used as a test response. The reproducibility of the test response was determined in three normal cats. It was found that the response remained constant for several hours. Reserpine (5 mg/kg) was administered i.v. When the acute blood pressure response to injection of reserpine had disappeared the sympathetic chains were stimulated alternately with a constant frequency (3, 5, 10 or 20 imp/sec) for one half to five minutes. Supramaximal voltage and impulse duration were used. A specific total number of impulses was thus applied. About 10 min after this total number had been delivered the test stimulus was applied. A ten minute interval was used since it was found that the test response in a non reserpinized cat was considerably diminished immediately after stimulation with a frequency of 20/sec for 5 min. *With an* interval of 10 min between the two types of stimulation, however, the test response was reproducible. The sequence of stimulations was repeated several times until no vasoconstrictor responses remained. The stimulation frequencies

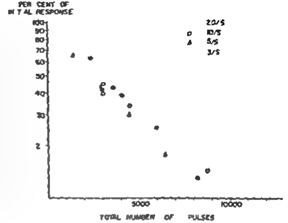

Fig 3 Percentual change in test response after increasing number of stimuli delivered to the sympathetic outflow following reserpine administration. Ordinate Peripheral resistance in percent of initial test response Logarithmic scale Abscissa Total number of stimuli delivered in the sympathetic outflow after reserpine administration (5 mg/kg i.v) Linear scale

Two experiments were performed with a frequency of 3 5 and 10 imp/sec respectively 4 experiments with 20 imp/sec For the test stimulus 10 imp/sec for 30 sec was invariably used

used varied from one experiment to another As a rule different frequencies were used for the two sympathetic chains Blood flow was recorded in both the left and right hind legs There seems to be a logarithmic relationship between the number of nerve impulses delivered after the administration of reserpine and the diminution of the test responses (Fig 3) This relationship seems to be independent of the frequency used The total number of impulses necessary for disappearance of the vasoconstrictor responses was the same irrespective of the frequency used

Discussion

As noted in the introduction the observations of numerous authors suggest that the depletion of catechol amines in the adrenal medulla after reserpine may be influenced by the nervous outflow With respect to the responses to adrenergic vasoconstrictor nerve stimulation the present experiments indicate a similar relationship Thus decentralization precludes to a large extent the disappearance of vasoconstrictor responses after reserpine (Fig 1) It is conceivable that the stimulation responses diminish in ratio to the depletion of transmitter substance from the nerves following reserpinization There is however, no direct evidence of such a relationship since no experiments correlating the vasoconstrictor nerve stimulation responses to the amount of transmitter substance available in the relevant peripheral organ seem to have been carried out MUSCHOLL and VOGT (1958) measured the noradrenaline content of rabbit superior cervical ganglion after reserpine treatment and found that severe noradrenaline depletion failed to impair the pupillary and palpebral responses to electrical stimulation of the cervical sympathetic chain The noradrenaline content of the superior cervical ganglion however is not necessarily comparable to that at the peripheral nerve endings

IGGO and VOGT (1960) have shown that reserpine does not alter the normal impulse discharge in sympathetic preganglionic nerves The part played by the central nervous system in the peripheral action of reserpine cannot therefore be attributed to an increased impulse frequency There remains the possibility that the normal impulse discharge may play an important role

Data accumulated during the last few years indicate that reserpine blocks a transport mechanism The results of HUGHES and BRODIE (1959), who used isolated blood platelets of guinea pigs indicate that 5 hydroxytryptamine, noradrenaline and adrenaline are taken up by the platelets via two processes — diffusion and active transport Reserpine seemed to inhibit the active transport and thus to block the concentrating mechanism KIRSHNER (1962) used isolated catechol amine granules from beef adrenal medulla and he too concluded that reserpine blocks the active transport of catechol amines The findings of EULER and LISHAJKO (1961) are directly relevant to our experiments since they pertain to isolated catechol amine granules of autonomic nerves (bovine splenic nerves) Under the prevailing experimental conditions there was a spontaneous release of noradrenaline from the granules This release was inhibited by small amounts of reserpine The *in vitro* experiments thus indicate that reserpine blocks the transport of catechol amines to or from storage granules The findings of BERTLER HILLARP and ROSENGREN (1960) may be similarly interpreted If reserpine s primary mechanism of action is the same *in vivo* the influence of the impulse discharge from the central nervous system on the depletion of catechol amines in peripheral organs after reserpine could possibly be accounted for If the transport mechanism which concentrates the substance at the nerve endings is blocked after reserpine, nerve would continue to release transmitter substance as long as any of the latter remained in store The size of the store prior to reserpine administration would thus determine the number of nerve impulses which could induce vasoconstrictor effects The degree of depletion of transmitter substance at the nerve endings after reserpine treatment would thus depend on the total number of nerve impulses delivered With this type of depletion a correlation between the decline of the responses to vasoconstrictor nerve stimulation and the total number of nerve impulses could also be expected Such a relationship apparently exists (Fig 3) When the percentual decrease in vasoconstrictor responses to a test stimulus is plotted on a logarithmic scale and the total number of impulses on a linear scale there seems to be a linear relationship between the total number of nerve impulses and the decline of the responses after reserpine

It is interesting to note that a total of about 10 000 impulses was necessary for the complete disappearance of the vasoconstrictor responses (Fig 3) According to FOLKOW (1952) the impulse frequency in vasoconstrictor nerves is around 2 imp/sec under resting conditions Thus 10 000 impulses would be produced in 1—2 hours Within that period as indicated in Fig 1 the responses to vasoconstrictor nerve stimulation had — in cases in which the sympathetic

nerves were intact — almost disappeared Thus the quantitative results obtained favour the idea that the depletion of transmitter substance after reserpine is mediated to a large extent by the normal nerve impulse discharge in the sympathetic nervous system HERTTING AXELROD and PATRICK (1962) and WEINER PERKINS and SIDMAN (1962) also suggested that the normal impulse discharge may play a role in the depletory action of reserpine

Does such a mechanism account for all the effects of reserpine on peripheral organs? Several authors have observed signs of a release of catechol amines on injection of reserpine MUSCHOLL and VOGT (1957) found an elevated blood level of adrenaline in rabbits within the first hour after an intravenous dose of reserpine (1—2 3 mg/kg) STJARNE and SHAPIRO (1958) measured in cats the outflow of catechol amines in the adrenal vein and recorded after reserpine (3 mg/kg i v) an increase in catechol amine output even from denervated adrenal glands The releasing effect might have been due to the relatively large doses of reserpine used EULER and LISHAJKO (1960) found that large doses of reserpine enhanced the spontaneous release of noradrenaline from isolated granules from bovine splenic nerves The decrease in vasoconstrictor effects even in the sympathetically denervated vascular bed (Fig 1) might thus be explained by the high dose (5 mg/kg i v) of reserpine used in our experiments Another possible explanation of the diminished vasoconstrictor responses in the denervated vascular bed could be a continuous slight leakage of transmitter substance from the nerve endings Provided no new transmitter substance is stored after reserpine such a leakage would lead to a gradual depletion of transmitter substance According to BURN and RAND (1958) a certain leakage of noradrenaline may occur normally Furthermore BURNSTOCK and HOLMAN (1962) found in isolated guinea pig vas deferens a spontaneous discharge of miniature junction potentials in the absence of nerve stimulation Such discharge is probably due to a spontaneous release of transmitter substance

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Acta physiol scand 1962 56 315—323

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Hemoglobin Oxygen Saturation in the Dog Kidney

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Abstract

AUKLAND K. *Hemoglobin oxygen saturation in the dog kidney* Acta physiol scand 1962 *56* 315—323 — Blood from the various zones of the intact kidney of anesthetized dogs was collected by puncture with glass capillaries and hemoglobin oxygen saturation percentage (% HbO_2) determined spectrophotometrically The mean values of 93 samples in 7 dogs from the renal cortex outer and inner medullary zone respectively were 68 65 and 45 % HbO_2 during air respiration and 83 71 and 47 % HbO_2 during oxygen respiration The mean cortical % HbO_2 was found to be 4 % HbO_2 lower than renal venous HbO_2. The mean urine oxygen tension during air respiration was 23 mm Hg and during oxygen respiration 33 mm Hg No correlation could be demonstrated between inner medullary HbO_2 and corresponding urine pO_2. It is concluded however that the low HbO_2 in the inner medulla supports the hypothesis that urine pO_2 is determined in the inner medulla by equilibration across the walls of the collecting ducts The slight influence of oxygen respiration on inner medullary HbO_2 may be due to a counter current effect of the vasa recta

The high oxygen content of renal venous blood (hemoglobin oxygen saturation approximately 80 %) suggests a high oxygen saturation in blood leaving the renal cortex since the cortical circulation probably accounts for nearly 80 % of the total renal blood flow (ULLRICH KRAMER and BOYLAN 1961) On the other hand several observations suggest a low hemoglobin oxygen saturation in medullary and especially papillary blood

1 Polarographic estimation of oxygen availability in the dog kidney indicated a lower oxygen tension in the renal medulla than in the cortex (AUKLAND and KROG 1960)

2 The low urine oxygen tension demonstrated by several investigators in dog and man (RENNIE, REEVES and PAPPENHEIMER 1958, HONG et al 1960, AUKLAND and KROG 1961, AUKLAND 1962 a) is thought to reflect a low oxygen tension in the renal papilla by equilibration across the walls of the collecting ducts.

3 High capacity for anaerobic glycolysis in tissue slices from inner medulla compared to that of cortex (CAPEK and KLEINZELLER 1961, KRAUS and ULLRICH cited by ULLRICH, KRAMER and BOYLAN 1961) might indicate a low availability of oxygen in the former area.

By contrast KRAMER, THURAU and DEETJEN (1960) found by oxymetric technique a hemoglobin oxygen saturation of 100 % in the inner medulla of dogs breathing pure oxygen, while urine oxygen tension would indicate a considerable unsaturation even during oxygen breathing. The present study was therefore undertaken to provide a direct estimate of hemoglobin oxygen saturation of blood from different zones of the kidney. An attempt was also made to correlate urine oxygen tension to medullary hemoglobin oxygen saturation.

While this study was carried out, Kramer and his group revised their previous conclusion and reported a considerable lower oxygen saturation in the renal medulla than in the cortex, in good agreement with the findings presented below (KRAMER, DEETJEN and BRECHTELSBAUER 1961, ibid cited by ULLRICH, KRAMER and BOYLAN 1961).

Methods

Eight experiments were performed in healthy mongrel dogs weighing 9—25 kg. Anesthesia was induced by Nembutal sodium i v 25 mg/kg body weight and maintained with subsequent doses of 1—2 mg/kg. A tracheal tube was inserted to assure free air ways. Oxygen was administered in some experimental periods by blowing 100 % oxygen into the tracheal tube *via* a polyethylene catheter. All experiments were performed with spontaneous respiration.

The left kidney was exposed retroperitoneally by a flank incision and the ureter catheterized by a polyvinyl catheter. The kidney was then gently dissected free and transposed into the flank incision, care being taken to avoid stretching or kinking of the renal pedicle. The kidney was then covered with gauze soaked in saline at 37 °C.

To secure a high urine flow 5 % mannitol in 0.45 % saline was infused i v at a constant rate of [illegible] ml/min. Urine oxygen tension (urine pO_2) was recorded continuously throughout 5 experiments with the technique described in previous papers (KROG and JOHANSEN 1959, AUKLAND and KROG 1961).

In one experiment renal venous blood was collected from a catheter in the left renal vein for determination of hemoglobin oxygen saturation. All contributaries to the renal vein had been ligated beforehand. Arterial blood was sampled from an indwelling catheter in the brachial artery.

The exposed kidney was punctured by means of heparinized glass capillaries with an outer diameter of 1.7 mm, inner diameter 0.6—0.7 mm and length approximately 150 mm. One end of the capillary had been tapered providing an outer diameter of approximately 0.5 mm at the tip. The capillary was inserted at the convexity of

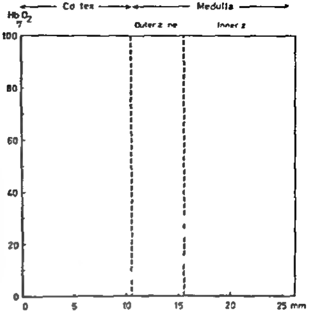

Fig 1 Hemoglobin oxygen saturation in blood from various zones of the left kidney of anesthetized dog Abscissa Depth below kidney surface in mm Open circles Oxygen respiration Filled circles Air respiration, Blood samples hemolyzed by freezing

the kidney and directed against the papilla, the free end being closed by the finger during introduction The depth of the puncture was chosen before each sampling and marked on the capillary with a skin pencil. With the capillary tip positioned in the renal cortex rapid filling was obtained without suction In the inner medulla however capillary force was often insufficient in filling the capillary and gentle suction by mouth through a polyethylene tubing attached to the capillary was necessary During sampling or immediately afterwards the blood sample was mixed by a small iron wire which was introduced into the capillary and moved to and fro by a magnet outside the capillary (AUKLAND 1962 b

The kidney was removed after each experiment and the width of the cortex inner and outer medulla was measured The puncture channels visibl by extravasation of blood were checked to assure that the deepest punctures had really been in the inner zone of medulla.

Hemoglobin oxygen saturation was determined spectrophotometrically according to the principle of DRABKIN and SCHMIDT 1945 A micro modification of the technique has been described elsewhere (AUKLAND 1962 b) In 5 experiments the blood was hemolysed by introduction of 2—3 mm of 10 saponin (Merck) into the glass capillaries and mixing by iron wire and magnet The standard deviation with this technique was as high as 4 % HbO possibly due to incomplete hemolysis In the remaining three experiments freezing was used for hemolysis giving a standard deviation of about 0.5 % HbO_2 (AUKLAND 1962 b

Frequent occurrence of clotting during sampling especially in the inner medulla, made it necessary to investigate if coagulation *per se* would introduce any systematical error in the determination of HbO_2 saturation A venous sample was drawn into a 20 ml glass syringe mixed thoroughly and then placed in ice water During clotting glass capillaries were filled anaerobically from the syringe providing a series with

Table I Hemoglobin oxygen saturation of renal blood in 7 dogs

	Air respiration			Oxygen respiration		
	Cortex	Medulla		Cortex	Medulla	
		Outer	Inner		Outer	Inner
Number of observations	27	10	14	21	11	10
Mean of all observations	67.6	65.1	44.6	83.2	71.1	46.6
SE of mean % HbO_2	1.89	4.60	4.40	2.23	3.63	5.91
Average of individual mean values, % HbO_2	67.4	63.7	50.7	84.3	72.6	48.8

decreasing hematocrit down to 1/20 of the initial value. Several experiments of this kind with varying hemoglobin oxygen saturation showed that partial clotting of the sample did not introduce any systematical error. As would be expected, however, the reproducibility was slightly poorer at low readings.

Results

Hemoglobin oxygen saturation in renal blood

Hemoglobin oxygen saturation (HbO_2) was determined in a total of 93 samples from the left kidney of 7 different dogs, 42 samples obtained during air respiration and 51 during oxygen respiration. As noted above, cortical ... were obtained easily without suction and without clotting. On the ... hand, gentle suction was often necessary for obtaining blood from the ...lla. In spite of constant stirring during sampling some degree of clotting often occurred during sampling from the medulla, resulting in hematocrit values as low as one tenth of that of systemic blood in some samples. Admixture of tissue fluid or preurine might probably also have contributed to the low hematocrit.

A representative experiment is shown in Fig. 1 where the HbO_2 values have been plotted according to depth below the kidney surface. In this experiment HbO_2 is clearly lower in the inner medulla than in the cortex, and seems to decrease towards the tip of the papilla. Qualitatively the same result was obtained in all experiments both with air and oxygen respiration except in one dog on air respiration, where a single value from the inner medulla exceeded the mean value for cortical samples (Fig. 2 a and b crosses). The mean values and standard error of mean are presented in Table I. (Due to the variable number of observations from each zone in different experiments slightly different mean values are obtained if the average of individual mean values are calculated, cf. Table I.) A composite graph of all observations is shown in Fig. 2 a and b. In this diagram the relative localization within each

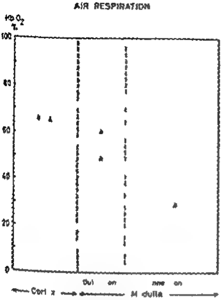

Fig 2 a

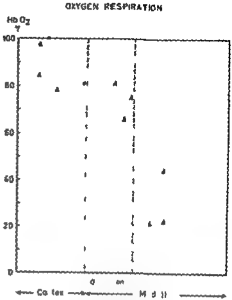

Fig 2 b

Fig 2 a Hemoglobin oxygen saturation in renal blood from 7 dogs breathing air Each symbol represents one animal The relative localization within each zone indicated only approximately

Fig 2 b Hemoglobin oxygen saturation in renal blood from 5 dogs breathing pure oxygen The same symbols for each dog are used in Figs 2 a and b

zone is only approximate It should be noted however that the majority of samples from the inner medulla were derived from the outer half of this zone

The HbO_2 values for the medulla showed far greater scattering than those from the cortex However statistical analysis showed a significant difference between cortex and inner medulla both during air and oxygen respiration although the evaluation is complicated by individual differences Oxygen respiration caused a highly significant increase of cortical HbO_2 but no significant alteration of inner medullary HbO_2 Thus the difference between cortex and medulla was considerably greater during oxygen respiration than during air respiration

Renal cortical blood compared to arterial and renal venous blood

The mean oxygen saturation of cortical samples (Table II) was lower than that generally found in renal venous blood while identical or even higher values would be expected in the absence of an Oxford shunt (TRUETA *et al* 1947) In one experiment a series of arterial renal venous and cortical blood samples were collected in the course of approximately one hour The mean cortical oxygen saturation was found to be 4 4 % HbO_2 lower than that of renal venous blood As evident from Table II cortical HbO_2 showed

Table II. Hemoglobin oxygen saturation percentage in blood from brachial artery, renal vein and renal cortex

	Brachial artery	Renal vein	Renal cortex
Number of samples	8	6	14
Mean	91.9	80.4	76.0
Range	90.9—93.2	78.[illegible]—82.6	63.9—82.[illegible]
S.E. of mean	0.27	0.70	1.52

a far greater scattering than that of venous HbO_2. However, 8 of the 14 cortical samples deviated by less than 2 % HbO_2 from renal venous HbO_2, while the remainder 6 deviated by more than 6 % HbO_2. Cortical HbO_2 did not exceed renal venous HbO_2 in any simultaneous samples.

Urine oxygen tension

Urine oxygen tension (urine pO_2) was recorded continuously throughout 5 experiments. Great spontaneous variations occurred both during air and oxygen respiration. Furthermore, it was found that even slight manipulation of the kidney tended to increase urine pO_2 for several minutes. However, puncture of the kidney with glass capillaries rarely influenced urine pO_2 in any direction. The effect of changing between air and oxygen respiration was variable and a new stable level of urine pO_2 was often reached after one or more cycles of increase and decrease. Urine pO_2 values corresponding to each blood sample taken from the kidney had the following average and range: Air respiration 23 (6—40) mm Hg. Oxygen respiration 32.6 (8—50) mm Hg. In any particular experiment no correlation could be established between urine pO_2 and corresponding hemoglobin oxygen saturation in the inner medulla.

Discussion

The method used for estimating hemoglobin oxygen saturation in renal blood samples is easily calibrated and has a well defined reproducibility. On the other hand, the blood sampling procedure deserves some comments. It might be expected *a priori* that blood obtained by direct puncture of a parenchymatous organ would have an oxygen saturation somewhere between arterial and venous blood from that organ. It was found, however, that cortical samples on the average had a lower oxygen content than renal venous blood. Since a high proportion of the cortical samples were very close to renal venous saturation, it is unlikely that the lower values are due to medullary shunting of blood (TRUETA *et al.* 1947), as also found by reflectometric determination of cortical oxygen saturation (MUNCK, LASSEN and KRAMER 1962). It seems more likely that the introduction of the glass capillary might produce local vaso-

spasm and ischemia, and thereby a fall in oxygen saturation during sampling. Admixture of blood from capsular veins is unlikely since most cortical samples were obtained 3—5 mm below the kidney surface. It seems reasonable to conclude that venous HbO_2 of the different zones are actually slightly higher than those obtained with the present technique. The localization of the capillary tip during sampling was well assessed by measurement of the depth below the kidney surface. It cannot be excluded, however, that blood might ooze along the capillary from more superficial zones and thus give erroneously high values in medullary samples.

The admixture of tissue fluid and/or preurine to medullary samples introduced technical difficulties by initiating clotting during sampling. However, since appreciable oxygen tension gradients between blood, interstitial fluid and tubules is unlikely (RENNIE *et al.* 1958), this should not alter the hemoglobin oxygen saturation. On the other hand, the low hematocrit of medullary samples does not indicate the existence of a red cell shunt in the outer medulla.

The low oxygen saturation in the inner medulla (average 44.6 % HbO_2 during air breathing) agrees well with the 49 % obtained with oximeter technique by KRAMER, DEETJEN and BRECHTELSBAUER (cited by ULLRICH, KRAMER and BOYLAN 1961). The considerably higher cortical saturation found by these investigators (92 %) may be due to a greater influence of arterial oxygen saturation on this method, and also to the factors tending to give too low values by the present method as discussed above. Furthermore, it has been observed repeatedly that the arterial oxygen saturation in anesthetized dogs may be as low as 85—90 % HbO_2, and this may partly explain the great scattering of individual mean values.

The inner medullary HbO_2 corresponds well to the average urine pO_2 of 22 mm Hg during air respiration (BARTELS and HARMS 1959). On the other hand, inner medullary HbO_2 during oxygen respiration (46.6 % HbO_2) was considerably lower than would be predicted from the average urine pO_2 (33 mm Hg). However, when the great fluctuations in urine pO_2 and the errors inherent in blood sampling are borne in mind, it must be concluded that the results support the hypothesis that urine pO_2 is determined when transversing the inner medulla through the collecting ducts. The lack of correlation of urine pO_2 to corresponding blood samples may be due to the variable origin of individual samples within the inner medulla.

It has been suggested that a low medullary oxygen content might result from a counter current effect of the vasa recta causing diffusion of oxygen from the descending to the ascending vessel limb (LEVY and SAUCEDA 1959, ULLRICH 1959, HONG *et al.* 1960, AUKLAND and KROG 1961, ULLRICH, KRAMER and BOYLAN 1961).

The anatomical substrate for a countercurrent exchange mechanism has been excellently demonstrated by TRUETA *et al.* (1947): the efferent arterioles of juxtamedullary glomeruli divide into the vasa recta which enter straight

into the medulla where they form sharp hairpin bends at varying levels in the outer or inner medulla Furthermore the arterial and venous limbs of the vessels are grouped together in bundles, especially in the outer medulla, giving a perfect countercurrent exchange system The influence of blood flow, oxygen consumption and diffusion properties has been discussed elsewhere (AUKLAND and KROG 1961, ULLRICH, KRAMER and BOYLAN 1961) and the arguments will not be repeated here However, the influence of varying arterial oxygen tension deserves some comments Assuming constant medullary oxygen consumption oxygen respiration will raise arterial and medullary venous oxygen saturation by the same amount Due to the S shaped oxygen saturation curve, however the arterial pO_2 will increase more than medullary venous pO The oxygen tension gradient between descending and ascending limb of the vasa recta is thereby increased, and presumably also the shortcircuiting of oxygen in the outer medulla The rise in inner medullary oxygen concentration would therefore be expected to be lower than the rise in arterial (and cortical) oxygen concentration, in agreement with the present findings KRAMER DEETJEN and BRECHTELSBAUER (cited by ULLRICH KRAMER and BOYLAN 1961) found considerably greater increase of inner medullary oxygen saturation on oxygen respiration (49—75 %) and suggested that this might result from a red cell shunt in the outer medulla (ULLRICH PEHLING and STÖCKLE 1961) Further more RENNIE *et al* (1958) found higher mean values for urine pO_2 than in the present study and also a greater effect of oxygen respiration The discrepancy may be due to different experimental conditions The hypothesis set out previously (AUKLAND and KROG 1961) that an increase of sodium load to the medulla increases medullary oxygen consumption might explain the low oxygen tension in the present experiments which were all performed during osmotic diuresis If under these circumstances the medullary metabolism is mainly or partially anaerobical an additional supply of oxygen might increase medullary oxygen consumption without appreciably influencing medullary and urine oxygen concentration It should be emphasized however that papillary oxygen saturation cannot be predicted from urine pO_2 with any accuracy as long as the pH of the papillary blood and interstices is unknown

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Acta physiol scand 1962 56 324—333

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Relative Adrenaline Content in Brain Tissue

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Abstract

GUNNE L M *Relative adrenaline content in brain tissue* Acta physiol scand 1962 *56* 324—333 — Following chromatographic separation on a weak cation exchange resin (Amberlite IRC 50) adrenaline was identified in the brains of hens pigs and rats by means of 1) the ethylene diamine condensation method 2) the trihydroxyindole method and 3) bioassay on the hen s rectal caecum and cat s blood pressure The percentage adrenaline in these species varied from 4 5 to 17 In brains of oxen and guinea pigs no adrenaline was detected indicating less than 4 per cent adrenaline in these species

In 1946 EULER reported the presence of sympathin in extracts from mammalian brains HOLTZ (1950) confirmed this observation and found that adrenaline represented about 10 per cent of the sum of adrenaline and nor adrenaline in ox and rabbit brains At this time the catecholamines of the brain were regarded as mainly belonging to the vasomotor nerves of the brain vessels

The situation became different when VOGT (1954) published her investigation on the distribution of the catecholamines in different parts of the mammalian brain The high concentrations in certain parts of the brain stem indicated a possible function of the amines in the nervous tissue itself

Since this time many papers have appeared dealing with the influence of various drugs on the catecholamines content of the central nervous system The relative contribution of adrenaline to the brain sympathin has however, been a matter of some controversy VOGT (1954) MONTAGU (1957) and BERTLER *et al* (1958) have used paper chromatography for separation of the catecholamines extracted from relatively small amounts of brain tissue combined

with sensitive methods of assay VOGT using bioassay recorded 5 per cent adrenaline in the hypothalamus of cats and 13 7 per cent in dogs MONTAGU found 34 per cent adrenaline in the rat brain while BERTLER and ROSENGREN (1959) and BERTLER (1960) were unable to detect significant amounts of adrenaline in mammalian brains SANO *et al* (1960) using columns of Amberlite IRC 50 for separation of the amines reported absence of adrenaline in the brains of various species of animals However they failed to detect adrenaline even in amphibians where large amounts have been found by other investigators (*cf* CARLSSON 1959)

Of the works reporting differential determinations of eluates containing the catecholamines in mixture only a few are cited here SHORE and OLIN reported (1958) a virtual absence of epinephrine in the rabbit brain stem by the trihydroxyindole method and spectrophotofluorometric technique while WEIL MALHERBE and BONE (1957) obtained 22 per cent adrenaline[1] in the same species by the ethylenediamine condensation method PAASONEN and DEWS (1958) recorded a mean level in the rat brain of 9 2 per cent adrenaline by bioassay KOVACS and FAREDIN (1961) reported a 10 % adrenaline[1] in the hypothalamus of rats determined by fluorimetric methods and STUFFEL and ROFFI (1961) found 7 5 per cent adrenaline[1] in rat brains using a similar method GUNNE (1959) found 4 3 per cent adrenaline in the rat brain using bioassay and 4 2—5 3 per cent with a fluorimetric assay method (1962)

The present paper deals with the relative amounts of adrenaline and noradrenaline in the brains of different species of animals In order to compensate for the difficulties presented by the small amounts of adrenaline a considerable number of brains were pooled from each species As the concentration and purification steps include considerable losses a starting level of 100—200 μg of noradrenaline was aimed at before a chromatographic separation of the amines was undertaken using a column of the weak cation exchange resin Amberlite IRC 50 The results obtained were compared with the routine determinations of eluates containing mixtures of the catecholamines according to EULER and LISHAJKO (1961) A preliminary report of part of this work has been given earlier (Gunne 1962)

Methods

The hypothalami of 100 pigs were homogenized and extracted with 5 volumes of 0 4 N perchloric acid After centrifugation the supernatant was titrated to pH 4 with 5 N KOH and the potassium perchlorate was spun down After adjustment of pH to 8 4 (addition of the disodium salt of ethylendiaminetetraacetate, 5 mg/g wet tissue weight prevented flocculation at pH 7—8) about 500 ml of the extract was passed through a column of 4 g alumina and eluted with 5 ml 1 N acetic acid followed by ml 0 25 N acetic acid When the whole extract had been treated the same way the eluates were pooled and 1/2 ml was taken for a preliminary assay according to EULER and LISHAJKO (1961) The rest of the eluate about 60 ml was concentrated to dryness *in vacuo* After

[1] percentages calculated from figures given in the text.

Table I Adrenaline and noradrenaline in pooled eluates of brain tissue from different species Extract is purified on alumina fluorimetric estimation by the THI method using two sets of filters

Species	Part of brain	Total amount in pooled eluates		Per cent Adrenaline
		Noradrenaline µg	Adrenalin µg	
Hen	Brain stem	108	22	17
Pig	Hypothalamus	86	7 0	7 5
Rat	Brain stem	102	4 8	4 5
Ox	Hypothalamus	206	0	<4
Guinea pig	Total brain	230	0	<4

dissolving in 2 ml of distilled water the pH was adjusted to 6 4 with 0 2 M ammonia A small precipitate was spun down and the supernatant was placed on a column of Amberlite IRC 50 measuring 500 × 5 5 mm Elution was performed according to KIRSHNER and GOODALL (1957) with 0 4 M ammonium acetate buffer pH 5 1 3 ml fractions were colllected and 0 5 ml of each fraction was treated by the ethylene diamine condensation method of WEIL MALHERBE and BONE (1952) The fluorescence of the fractions was measured in a Coleman 12 C fluorimeter using a filter with a maximum transmission at 436 nm for the excitation light and a filter with transmission limit at about 500 nm for the fluorescence light The fluorescence intensity of the ethylene diamine condensate of dopamine is about 1/5 and the condensate of adrenaline about 1/3 of that of noradrenaline using these filters

One sample containing the hypothalami of 50 ox brains one containing the brain of 300 rats and one the brain stems of 240 hens were treated in the same way A le containing the whole brains of 400 guinea pigs had to be purified further to d damage to the amberlite column The dried eluate was washed with 30 ml of methanol (8 ml N sulphuric acid in 30 ml methanol) After centrifugation the extract was concentrated to dryness *in vacuo* and treated as described above The brains from the hens and the guinea pigs were received 2 –4 hours *post mortem* while the rest were extracted within minutes after killing

The fluorescence peaks found were identified further in the following ways 1) The Rf values of standard solutions containing 5 µg adrenaline 50 µg noradrenaline and 50 µg dopamine dissolved in 2 ml of an 0 2 M ammonium acetate buffer pH 6 1 to 6 6 were studied in the same Amberlite IRC 50 column system 2) The activation and fluorescence peaks of the ethylene diamine condensates were determined in an Aminco-Bowman spectrophotofluorometer and compared with known standard solutions 3) The rests of the fractions which had not been used for ethylene diamine condensation were pooled so as to make 2 new samples corresponding to the A and the first part of the NA + DA peaks The samples were adjusted to pH 8 4 and adsorbed on a alumina column Elution was performed with 0 25 N sulphuric acid and after neutralization the biologic effects were tested on the cat s blood pressure and the hen s rectal caecum The cats were pretreated with the drug combination recommended by KARKI (1956) (atropine sulfate 2 mg/kg subcutaneously cocaine hydrochloride 8 mg/kg ergotamine tartrate 0 15 mg/kg and mepyramine maleate 3 mg/kg intramuscularly) which rendered them extremely sensitive to noradrenaline When biological effects had been established they were abolished by oxidation in alkaline solution One part of each eluate was treated by the trihydroxyindole method and a *fluorimetric*

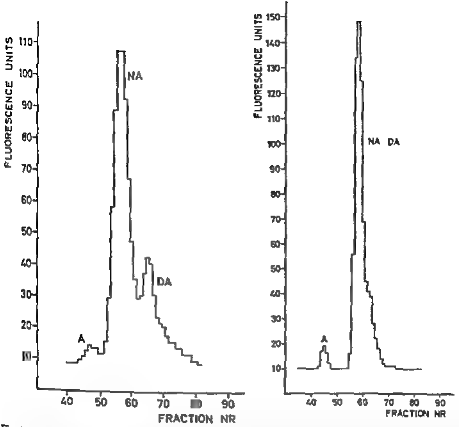

Fig 1 Separation of 5 μg adrenaline (A) 50 μg noradrenaline (NA) and 50 μg dopamine (DA) on Amberlite IRC 50 Column 500 × 5.5 mm equilibrated with a 0.2 M ammonium acetate buffer at pH 6.1 (left) and pH 6.6 (right) Elution with a 0.4 M ammonium acetate buffer at pH 5.0 Fraction size 0.8 ml.

determination was made according to EULER and LISHAJKO (1961) on a Coleman fluorimeter using two sets of filter combinations 4) In order to achieve an optimal separation of adrenaline and noradrenaline the demands for a complete separation of noradrenaline and dopamine had to be reduced The admixture of dopamine in the noradrenaline fractions could however be determined by the method of CARLSSON and WALDECK (1958)

Results

Standard solutions

Solutions containing 5 μg adrenaline and 50 μg each of noradrenaline and dopamine gave different fractionation curves depending on pH at application to the Amberlite IRC 50 column In accordance with the observations of KIRSHNER and GOODALL (1957) the lower pH 6.1 gave a better separation between noradrenaline and dopamine A higher pH 6.6 increased the distance between adrenaline and noradrenaline while in this case the dopamine fluo-

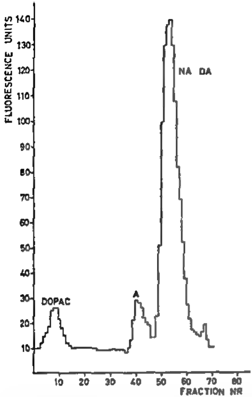

Fig 2 Separation of concentrated eluate from the brain stems of 240 hens on Amberlite IRC 50

..cence was covered by the noradrenaline curve (Fig 1) Pilot studies indicated ..at 2 μg of adrenaline (corresponding to 4 per cent provided the noradrenaline content is 50 μg) represented the lowest amount that could be detected by this method

Fluorimetric determination of eluates containing the catecholamines in mixture

Table I gives the results of the preliminary fluorimetric estimation of noradrenaline and adrenaline in a small part of the initial eluates of brains from different species, using the method described by EULER and LISHAJKO (1961) As evident from these values the percentage of adrenaline seems to vary from one species to another

Chromatographic separation of eluates on Amberlite IRC 50

The fractionation curves obtained from the main part of the eluates by passage of the concentrated sample through an Amberlite IRC 50 column, gave further support to the observed species difference

The high osmolarity of the brain tissue extracts probably interfered with the adsorption of the amines to the top of the amberlite column and in addition caused some damage to the structure of the resin resulting in a somewhat shorter effective column These factors diminished the resolution capacity of the columns to some extent

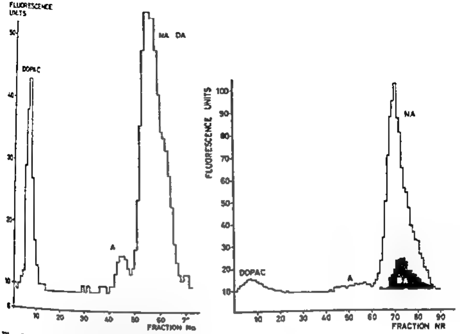

Fig 3 Separation of concentrated eluate from hypothalamus of 100 pigs (left) and from brain stems of 300 rats (right) on Amberlite IRC 50

The size of the adrenaline peaks (A) of Fig 2—4 agree well with the relative amounts of adrenaline of Table I The ethylene diamine condensates of the adrenaline (A) fractions of brains from hens pigs and rats had fluorescence and activation peaks identical with those of similarly treated standard solutions (activation peak 400 nm fluorescence peak 540 nm[1]) when measured in the spectrophotofluorometer The noradrenaline fractions gave fluorescence maxima which could be differentiated from adrenaline (activation peak 410 nm fluorescence peak 510 nm [1])

A rapidly moving fraction containing acid products appeared in the first 10—15 fractions of the eluate The fluorescence and activation maxima of the ethylene diamine condensation product of this peak (measured in an Aminco-Bowman spectrophotofluorometer) agreed with that of 3 4-dihydroxyphenyl acetic acid (DOPAC) and differed from adrenaline and dopamine (*cf* KAGI BURGER and GIGER 1957) Although this peak may have contained also other acids and no other measures of identification were undertaken is was for convenience termed DOPAC (Fig 2—4)

uncorrected instrumental values

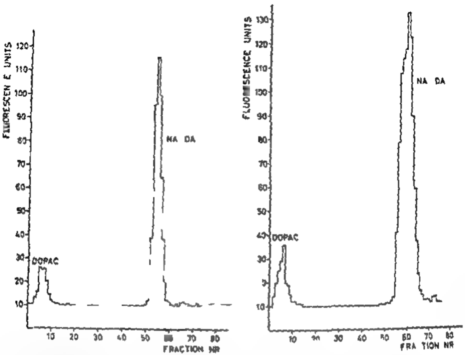

Fig 4 Separation of concentrated eluate from hypothalamus of 50 oxen (left) and from whole brains of 400 guinea pigs (right) on Amberlite IRC 50

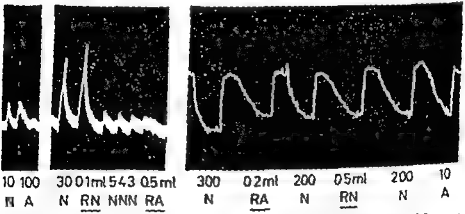

Fig 5 Bioassay of adrenaline (RA) and noradrenaline (RN) derived from separated fractions of rat brain (cf Fig 3) Doses of standards noradrenaline (N) and adrenaline (A) are given in ng
Left assay on cat's blood pressure 1 ml RA equals 0.001 μg N 1 ml RN equals 0.4 μg N Activity ratio A : N = 0.1
Right assay on hen's rectal caecum 1 ml RA equals 1 μg N 1 ml RN equals 0.4 μg N Activity ratio A : N = 15

Bioassay and fluorimetric (THI) determination of the fractions

The biological activity of the adrenaline fractions corresponded to that of pure adrenaline solutions when tested on the cat's blood pressure and the hen's rectal caecum (Fig 5) The activity was abolished by oxidation in alkaline solution When the THI method was used for fluorimetric determination on a Coleman fluorimeter using two sets of filters the fluorescence agreed with pure adrenaline

The noradrenaline fractions of the rat brains were also determined for dopamine content according to CARLSSON and WALDECK 1958 (Fig 3 right)

Discussion

The parenteral administration of adrenaline in man is connected with several effects of possible central origin while corresponding amounts of noradrenaline are remarkably void of such action (for a review see ROTHBALLER 1959) Although noradrenaline and dopamine are the dominating catecholamines in mammalian brains the central effects of exogenous adrenaline suggest that even small amounts of adrenaline present in the nervous tissue might be of physiological importance However the almost complete blood brain barrier to catecholamines presents difficulties in the interpretation of results obtained by administration of the amines

The fluorescence peaks obtained mainly by noradrenaline in the brain eluates (Fig 2—4) after separation on Amberlite IRC 50 were approximately equal to those found with 50 μg of a standard solution of noradrenaline The only exception was the pig brain sample which contained about half as much noradrenaline as the others As stated above these amounts of catecholamines limit the resolution capacity of the method to about 4 per cent of adrenaline

The findings reported here indicate the presence of adrenaline in amounts exceeding 4 per cent in the brains of hens pigs and rats Regarding the high adrenaline content of the hens one is reminded of the phylogenetic relationship between birds and amphibians where adrenaline is known to predominate (CARLSSON 1959)

In the brains of oxen and guinea pigs adrenaline if present amounted to less than 4 per cent The species differences found in routine analyzes using a Coleman fluorimeter with two sets of monochromatic filters were reasonably well reflected by peaks of corresponding heights after chromatographic separation on Amberlite IRC 50 This observation strongly supports the validity of the adrenaline values obtained by differential estimation of eluates containing the catecholamines in mixture

The presence of 3 4-dihydroxyphenyl acetic acid DOPAC in the brain has been reported by EULER (1958) and the relationship between this substance and dopamine was studied by ROSENGREN (1960) who reported the presence of DOPAC in brains from man rabbits and pigs In all the brains studied here there was a peak with fluorescence properties identical with those of DOPAC

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Blood Coagulation Studies in Hedgehogs, in a Hibernating and a Non-Hibernating State, and in Dogs, Hypothermic and Normothermic

By

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Abstract

BIORCK, G B W JOHANSSON and I M NILSSON *Blood coagulation studies in hedgehogs in a hibernating and a non hibernating state and in dogs hypothermic and normothermic* Acta physiol scand 1962 *56* 334—348 — Different coagulation factors were studied in hedgehogs during hibernation and during the non hibernating state The dog was chosen as a non hibernator It was studied before cooling at a body temperature of 30 C and 25 C during cooling at 30 C during rewarming just after rewarming and at different times after rewarming The results are given in the tables The hedgehogs showed a prolonged coagulation and recalcification time during hibernation compared with the values observed in non hibernating animals The prothrombin two-stage test and the prothrombin and factor VII determinations showed lower values in late August than in May—June The antithrombin activity was increased in the hibernating hedgehogs compared with the non hibernating animals No circulating anticoagulant and no fibrinolysis were demonstrable in the hedgehogs In the dogs the prothrombin and factor VII values decreased in the later phase of the rewarming and remained at a low level during the first few days after rewarming The following days these factors increased to levels above the original ones The antithrombin activity showed a marked rise after rewarming In the rewarming phase there was in six dogs an increased fibrinolytic activity

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The first systematic studies of hypothermia were performed about a decade ago Since this time hypothermia has come into common use and it has turned out to be a valuable tool The physiology of hypothermia is however many respects quite different from that of the normothermic state and although many biochemical and physiological studies have been performed on hypothermic organisms many problems remain to be solved In the cardiological laboratory in Malmo we have studied the different aspects of function in the hypothermic heart (BJORCK and JOHANSSON 1955 JOHANSSON *et al* 1956) the immunological response (HALL and JOHANSSON 1961) and the histological changes in hypothermia (HALL *et al* 1960)

A review of the literature on coagulation changes during hypothermia reveals rather conflicting results

The *coagulation time* has been determined by many authors in different animals and with varying technique Many authors obtained a prolongation of the clotting time (WILLSON MILLER and ELIOT 1958 ROSS 1954 and DETERLING *et al* 1955) FISHER *et al* (1955) and GRAY (1957) in their long term experiments with cooled dogs found that a prolongation of the clotting time developed after some time FISHER *et al* (1955) wrote that by the fourth hour all animals demonstrated significant increase in coagulation time TERZIOGLU and ÖZER (1956) found a prolongation of the coagulation time in hypothermic cats while ELLIS and his associates (1957) found no change in coagulation time when dogs were subjected to hypothermia alone but a rather pronounced shortening when the animals underwent circulatory occlusion and open cardiac surgery in the hypothermic state In neurosurgical patients operated upon under hypothermia BUNKER and GOLDSTEIN (1958) observed a prolongation of the clotting times in all coagulation tests except whole blood clotting REED and GAVRILESCU (1958) also working in a neurosurgical unit were not able to demonstrate any significant changes in clotting times

SUOMALAINEN and LEHTO (1952) observed a prolongation of the coagulation time in hibernating hedgehogs and a similar result was found in hibernating bats (SMITH LEWIS and SVIHLA 1954) hamsters (SVIHLA, BOWMAN and PEARSON 1952 RATHS and PERLICK 1953) and ground squirrels (SVIHLA BOWMAN and RITENOUR 1951)

VON KAULLA and SWAN (1958) reported a tendency to shortening of the *recalcification time* of the human patients operated upon in hypothermia

TERZIOGLU and ÖZER (1956) found no definite changes in the *prothrombin time* in hypothermic cats and FALKMER and KJELLGREN (1955) could not find any change in prothrombin index or prothrombin proconvertin index in hypothermic rabbits DETERLING *et al* (1955) saw no variations in prothrombin time in dogs LASCH *et al* (1959) obtained an increase of prothrombin while ELLIS *et al* (1957) found a decrease When keeping dogs at a body temperature of about 23 C FISHER *et al* (1955) noted a prolongation of the prothrombin time but in man REED and GAVRILESCU (1958) observed no significant changes

When comparing active and dormant ground squirrels SVIHLA *et al* (1952) noted a reduction in the amount of prothrombin in the blood of dormant squirrels RATHS and PERLICK (1953) reached the same result in hibernating hamsters

The results in the literature on *factor V* concentration are contradictory LASCH *et al* (1959) noted an increase of the activity of factor V while ELLIS *et al* (1957) found that the factor V decreased No major changes in factor V were obtained in man by BUNKER and GOLDSTEIN (1958)

RATHS and PERLICK (1953) obtained low factor V values during hibernation in hibernating hamsters while no changes were found between hibernating and non hibernating hamsters

Prothrombin consumption time was shorter during hypothermia than before cooling as noted by WILLSON *et al* (1958) In man VON KAULLA and SWAN (1958) concluded that the prothrombin consumption reflected by the longer prothrombin serum times, was more complete in the specimens incubated at 28 C than in those incubated at 37 C but generally cooling failed to bring about any reduction of the prothrombin consumption below the normal values at 37 C In hypothermic neurosurgical patients BUNKER and GOLDSTEIN (1958) found a depression of prothrombin consumption

Lowering of the body temperature of cats markedly augmented the *antithrombin* activity of blood (TERZIOGLU and ÖZER 1956) TERZIOGLU and ÖZER also claimed to have established the identity of antithrombin as heparin During cooling of dogs LASCH *et al* (1959) observed a shortened clotting time which among other things was ascribed to a decreased antithrombin titre After clamping of the circulation for about an hour and cardiac massage during rewarming the clotting time was prolonged by an increase in heparin concentration

RATHS and PERLICK (1953) and SUOMALAINEN and his collaborators (1956) working respectively on hamsters and hedgehogs reported a marked increase of the antithrombin titre Both these teams conclude from experiments with protamine sulphate titrations that the antithrombin is mainly heparin SUOMALAINEN *et al* have also observed an increased amount of mast cells in the hedgehog during hibernation (SUOMALAINEN and LEHTO 1952 SUOMALAINEN and HÄRMA 1951) On the other hand SMITH *et al* (1954) using both summer and winter bats could not obtain good correlation between mast cell counts of duodenal tissues and blood clotting times

ELLIS *et al* (1957) noted a decrease of the *fibrinogen* content in dogs BUNKER and GOLDSTEIN (1958) observed a slight decrease of their fibrinogen values while REED and GAVRILESCU (1958) and KENYON *et al* (1959) found no change VON KAULLA and SWAN (1958) demonstrated an increased *fibrinolytic* activity in some of their patients

From the foregoing it can be seen that the coagulation mechanism during hypothermia has been studied by many authors but the results differ widely It is however a definite clinical impression that during surgical operations in

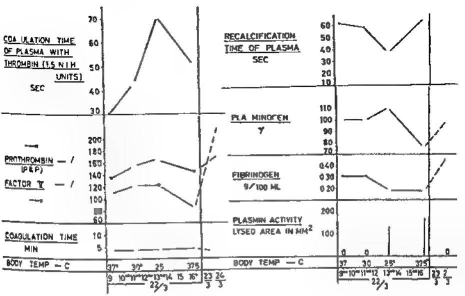

Fig 1 Changes in different coagulation factors during hypothermia in a dog See further the text

hypothermia there is sometimes an increased bleeding tendency (ELLIS KLEIN SASSER and SPEER 1957 WENSEL and BIGELOW 1959 GRAY 1957 WADDELL FAIRLEY and BIGELOW 1957) Because of the importance of this clinical finding we deemed it valuable to elucidate the changes in the different blood coagulation factors during hypothermia From the beginning of our studies in hypothermia we have been interested in hibernation and whenever possible have tried to compare the corresponding parameters of a hibernator and a non hibernator We have followed this system in the present study with the dog and the hedgehog as experimental animals

Methods and material

Hedgehogs were obtained during the summer and kept in an out door stall during the winter Sixteen hedgehogs were examined in January—February (series A) 12 in late May—June (series B) and 7 in late August (series C) The hedgehogs in series A were hibernating with a body temperature of 5—13 C while the animals in series B and C were non hibernating and with a body temperature of 33—34 C After the thorax had been opened blood samples were taken from the aorta with silicon coated syringes Anesthesia was induced in the non hibernating animals with a small dose of sodium pentothal The hibernating animals needed no anesthesia

Eleven adult mongrel dogs were used whose weights varied from 12 to 23 kg The dogs were also anesthetized with sodium pentothal Artificial respiration was begun after cooling when the rectal temperature had fallen to about 32 C The technique has been described in detail in previous papers (WULFF *et al* 1957 HÆGER JOHANSSON and SJOSTROM 1957) After anesthesia all blood samples were drawn from the femoral

Table I Hedgehogs

Determination	Series A (January February)		
	No of animals	Mean	Range
Coagulation time in min at 18—20 C	5	11	9—14.5
Recalcification time of plasma in sec	9	164	101—270
One stage prothrombin time in sec	16	23	16—43
Prothrombin + factor VII (P+P) in per cent	16	13 2	4—70
Prothrombin (two-stage) — units per ml	4	55	46—70
Factor V in per cent	15	542	300—1 000
Fibrinogen in per cent	7	0 50	0 07—1 04
Fibrinolysis	16	0	—
Antithrombin			
Plasma + 15 N I H units of thrombin (sec)	6	27	13—41
Plasma + 6 N I H units of thrombin (sec)	6	65	29—114
Plasma + 3 N I H units of thrombin (sec)	14	422	50—1 000

vein with silicon coated syringes before cooling at 30 C at 25 C during cooling and at 30 C during rewarming A final sample was taken after the return of body temperature to normal values and in some cases samples were taken on one of the following days (1—11 days after cooling) Four dogs were kept for 6 hours 1 dog for 4 hours and 1 dog for 2 hours at the lowest temperature (about 26 C) Blood samples were taken at two-hour intervals during this time Three dogs were cooled twice with an interval of 5—6 weeks

Venipuncture was performed with sharp widebore silicon coated needles The first few millilitres of blood obtained were discarded and then allowed to flow directly through the needle into a series of tubes The following tests were made *coagulation time* (according to the method of HEDENIUS (1936) with the modification that the size of the tubes was 50 mm in height and 10 mm in diameter) *recalcification time* of plasma (NILSSON BLOMBÄCK and VON FRANCKEN 1957) *one stage prothrombin time* of plasma with human brain thromboplastin (OWREN 1947) *prothrombin and factor VII* (OWREN and AAS 1951) *prothrombin* (two-stage according to WARE and SEEGERS 1949) *factor V* (WOLF 1953) *prothrombin consumption test* (BIGGS and MACFARLANE 1957) — in the hedgehogs only in series B — *fibrinogen* (JACOBSSON 1955) *fibrinolysis* — different methods were used (a) determination of the time necessary to dissolve a clot after addition of thrombin to the blood at + 37 C (b) by a modification of Schneider's (1952) serial dilution method Citrated plasma was diluted in ratios from 1 10 to 1 320 and then clotted with thrombin (1 ml plasma in various dilution + 0 1 ml thrombin (Topostasine Roche) containing 100 N I H units/ml) The tubes were placed in a water bath at + 37 C then ten minutes after the addition of thrombin the highest plasma dilution showing a definite clot was read The dilutions were allowed to remain in the water bath for a further 2 hours and then again examined for the presence of a clot Any tube which had lysed completely over the 2 hour period was marked *plus* and since lysis was most apt to occur in the higher dilution a scoring for example of 3 plus means that the three highest dilutions have lysed within 2 hours (c) the fibrinolytic activity of plasma was also determined on unheated bovine Astrup fibrin plates in the way described by NILSSON *et al* (1960) The *antithrombin* activity of plasma was studied by

Series B (May June)			Series C (Late August)		
No of animals	Mean	Range	No of animals	Mean	Range
1?	5	3—7	6	4	3—?5
1?	108	8?—14	7	70	45—118
1?	2?	18 3—?6 9	7	2?	19 4—28
1?	17.3	10—?6	7	6 5	3 4—8.2
4	90	60—120	4	20	18—21
10	3?4	2?0—4?0	?	450	250—500
7	0.28	0 06—0 12	3	0 54	0 3?—0 90
1?	0	—	7	0	—
7	12	8 —18	7	14	8—18
7	25	17— 36	7	29	14—30
6	61	33—8?	7	128	42—19?

determination of the clotting time of plasma after addition of thrombin of various concentration (0 2 ml citrated plasma + 0 2 ml thrombin solution containing 15 6 3 and 1.5 N I H units/ml respectively) In some experiments the antithrombin studies included the ability of serum to inactivate different amounts of added thrombin and the thrombin generation test (NILSSON and WENCKERT 1954 BIGGS and MACFARLANE 1957) The *hepa in co-factor* content of plasma was studied *in the* hedgehogs using the method of BLOMBACK *et al* (1955 and 1957 The experiments in the dogs also included determination of *circulating anticoagulants* (HALLELL and NILSSON 1957) and test for *thromboplastin* activity (BLOMBACK and NILSSON 1957 in the plasma

The coagulation time in some of the hedgehogs was determined at a temperature of 18—20 C and in others belonging to series A also at 10 C All the remaining coagulation analyses were performed at 37 C Normal human plasma always taken from the same person was regularly collected at the same time as the hedgehogs plasma and used as standard for the coagulation tests in the hedg hogs Plasma from a normal dog was used as a standard for the different coagulation factor in the cooled dogs

Results

Hedgehogs

The results of the coagulation studies of the hedgehogs are presented in Table I from which it may be seen that they showed a prolonged coagulation time and recalcification time during hibernation when compared with the values observed in the non hibernating animals The coagulation time in one animal of series A was 10 min when determined at room temperature and 16 min when determined at hibernation temperature (10 C) The one stage prothrombin time was the same for the different series On the other hand the prothrombin two-stage test and the prothrombin and factor VII determina

Table II Dogs

Determination	After anesthesia before cooling			Cooling 30 C			Cooling 25 C		
	No of animals	Mean	Range	No of animals	Mean	Range	No of animals	Mean	Range
Coagulation time in min at 18—20 C	11	6.5	2.6—11.5	10	7.0	3.0—12.0	10	6.6	1.6—16.0
Recalcification time of plasma in sec	11	133	62—312	10	98	39—228	9	92	38—183
One stage prothrombin time in sec	11	8.8	7.0—10.8	9	9.0	8.0—10.2	10	9.9	8.2—12.2
Prothrombin + factor VII (P + P) in per cent	11	99	80—136	9	106	76—196	10	99	68—159
Factor V in per cent	10	100	100	8	115	100—130	10	101	77—120
Prothrombin consumption in per cent residual prothrombin	10	3.4	0—8.8	10	6.7	0—16.6	8	6.2	0—24
Antithrombin									
Plasma + 6 N I H units of thrombin (sec)	11	13.9	5.4—24.0	10	13.0	8.2—19.0	10	14.9	8.2—23.0
Plasma + 3 N I H units of thrombin (sec)	10	23.2	10.0—40.0	9	20.9	14.0—34.2	8	27.0	14.8—55.0
Plasma + 1.5 N I H units of thrombin (sec)	6	31.0	18.0—45.0	6	34.2	25.0—43.0	6	47.3	35.0—77.0
Fibrinolysis									
Fibrinogen in g per 100 ml	11	0.32	0.17—0.63	10	0.33	0.14—0.79	10	0.35	0.19—0.81
Schneider's test	10	1	0—3	10	0.6	0—2	10	1.6	0—3
Hematocrit	6	39.5	30.0—43.5	5	41.8	38.7—44.7	5	45.3	42.8—50.4

Rewarming 37°C			Just after rewarming			1—2 days after cooling			3—4 days after cooling			5—11 days after cooling		
No of animals	M	Range	No of animals	Mean	Range	No of animals	Mean	Range	No of animals	Mean	Range	No of animals	Mean	Range
2	7.5	4.5—10.3	7	3.8	1.1—8.0	7	3.6	0.5—8.8	6	4.2	1.9—7.6	4	3.5	1.0—7.0
2	137	103—170	8	113	46—231	4	111	45—239	6	153	74—238	5	129	78—203
4	10.0	9.8—10.2	8	10.4	8.7—12.8	5	10.7	8.8—13.8	6	8.9	7.0—11.0	4	9.2	7.2—10.0
1	64	64	8	70	56—90	5	85	52—160	4	113	70—160	4	120	100—160
2	[illegible]	60—76	8	83	47—135	5	101	60—125	5	129	70—175	3	149	104—180
2	3.0	0.2—4.9	6	3.7	0—12.3	3	0.8	0—2.1	4	2.0	0—4.9	3	1.7	0—3.1
4	10.0	9.8—10.2	8	17.3	9.8—27.2	4	11.5	6.8—17.0	6	14.0	7.8—[illegible]0	2	13.5	13.0—14.0
2	16.2	15.3—16.8	7	46.1	17.0—146.0	4	20.8	11.0—36.0	6	30.1	14.0—53.0	3	21.2	18.2—26.[illegible]
4	27.1	26.0—28.2	4	179	26.0—600.0	4	37.0	17.8—68.0	4	69.6	22.8—196.0	1	38	38
1	0.20	0.20	8	0.30	0.12—0.67	5	0.38	0.22—0.60	5	0.49	0.30—0.69	3	0.35	0.15—0.50
1	0	0	8	1.1	0—2	5	1.6	1—2	4	0.6	0—2	2	0.7	0—1
1	56.7	56.7	4	49.8	[illegible]8—59.9	2	38.4	34.4—42.4	2	37.4	29.9—44.8	2	37.0	36.5—37.4

Table II Dogs

Determination	After anesthesia before cooling			Cooling 30 C			Cooling 25 C		
	No of animals	Mean	Range	No of animals	Mean	Range	No of animals	Mean	Range
Coagulation time in min at 18–20 C	11	6 5	2 6–11 5	10	7 0	3 0–12 0	10	6 6	1 6–16 0
Recalcification time of plasma in sec	11	133	62–312	10	[illegible]	39–[illegible]28	9	92	38–183
One stage prothrombin time in sec	11	8 8	7 0–10 8	9	9 0	8 0–10.2	10	9 9	8 2–17 2
Prothrombin + factor VII (P + P) in per cent	11	99	80–136	9	106	76–196	10	[illegible]	68–15[illegible]
Factor V in per cent	10	100	100	8	115	100–130	10	101	77–120
Prothrombin consumption in per cent residual prothrombin	10	3 4	0–8 8	10	6 7	0–16 6	8	6.2	0–24
Antithrombin									
Plasma + 6 N I H units of thrombin (sec)	11	13 9	5 4–24 0	10	13 0	8 2–19 0	10	14 9	8.2–[illegible]3 0
Plasma + 3 N I H. units of thrombin (sec)	10	23 2	10 0–40 0	9	20 9	14 0–34.2	8	27 0	14.8–55 0
Plasma + 1 5 N I H units of thrombin (sec)	6	31 0	18 0–45 0	6	34.2	25 0–43 0	6	47.3	3[illegible] 0–7[illegible] 0
Fibrinolysis									
Fibrinogen in g per 100 ml	11	0 3[illegible]	0 17–0 63	10	0 33	0 14–0 79	10	0.35	0 1[illegible]–0 [illegible]
Schneider s test	[illegible]	1	0–3	10	0 6	0–2	10	1 6	0–5
Hematocrit	6	39.5	30 0–43 5	5	41 8	38 7–44 7	5	46.3	4[illegible].8–50 4

Rewarming 30 C			Just after rewarming			1—2 days after cooling			3—4 days after cooling			5—11 days after cooling		
N	M n	Range	No. of animals	Mean	Range	No of animals	Mean	Range	No of animals	Mean	Range	No of animals	Mean	Range
2	7.3	4.5—10 3	7	3 8	1 1—8 0	7	3 6	0 5—8 8	6	4.2	1 9—7 6	4	3 5	1 0—7 0
2	137	103—170	8	113	46—231	4	111	45—239	6	153	74—238	5	129	78—203
2	10 0	9 8—10 2	8	10 4	8 7—12 8	5	10 7	8 8—13 8	6	8 9	7 0—11 0	4	9.2	7.2—10 0
1	64	64	8	70	56—90	5	85	57—160	4	113	70—160	4	129	100—160
2	68	60—76	8	83	42—135	5	101	60—125	5	129	70—175	3	149	104—180
2	3 0	0.2—4 9	6	3 7	0—12 3	3	0 8	0—2 1	4	2 0	0—4 9	3	1 7	0—3 1
2	10 0	9 8—10.2	8	17 3	9 8—27.2	4	11 5	6 8—17 0	6	14 0	8—25 0	2	13 5	13 0—14 0
2	16.2	15.5—16 8	7	46 1	17 0—146 0	4	20 8	11 0—36 0	6	30 1	14 0—53 0	3	21 2	18.2—26.2
2	27 1	26 0—28.2	4	179	26 0—600 0	4	37 0	1 8—68 0	4	69 6	22 8—196 0	1	38	38
1	0.20	0.20	8	0 30	0 12—0 67	5	0 38	0.22—0 60	5	0 49	0 30—0 69	3	0 35	0 15—0.50
1	0	0	[illegible]	1 1	0—2	5	1 6	1—2	4	0 6	0—2	2	0 7	0—1
1	56 7	56 7	4	49 8	39 8—59 9	2	38 4	34 4—42 4	2	37 4	29 9—44 8	2	37 0	36.5—37 4

tions showed definitely lower values in late August (series C) than in late May–June (series B). The hibernating animals (series A) showed values in between those of series B and C. The factor V content in the different animals varied considerably and no significant differences between series A, B and C can be seen. The antithrombin activity, determined by thrombin titration of the plasma was increased in the hedgehogs in series A compared to the values in series B and C which did not significantly differ from each other. The thrombin times of the hibernating plasma samples did not shorten after addition of fibrinogen. When plasma from the hibernating animals were added to human plasma in a proportion of 1 : 10 this resulted in a prolongation of the thrombin time of the human plasma from 35 sec to 60 sec. Thrombin generation test performed on plasma from the different series also showed a significantly higher antithrombin activity in series A. The antithrombin III activity was studied by determination of the capacity of serum to inactivate added thrombin. Serum obtained during the hibernating period inactivated about two times more thrombin than that taken during late August. The heparin co-factor activity of plasma was found to be about twice that of human plasma during the hibernation period while its action corresponded to that of human plasma during the late August period.

Any circulating anticoagulant active in the first phase of coagulation could not be demonstrated in plasma from the hibernating hedgehogs. Hedgehog plasma for these tests was added to normal human plasma in recalcification system and also to the reaction mixture of the thromboplastin generation test. Protamine chloride in various concentrations did not shorten the coagulation time.

Only a few fibrinogen determinations were performed in which the values varied widely within each group. No fibrinolytic activity in the hedgehogs could be demonstrated on any occasion.

Dogs

The results of the coagulation studies in 11 experiments with hypothermic dogs are given in Table II. Fig. 1 demonstrates the course in one typical case.

The coagulation time as well as the recalcification time showed a tendency to shorten during the hypothermic state in some dogs but the mean values showed no significant changes. The one stage prothrombin time showed only small changes of no significance. The values for prothrombin and factor VII showed a decrease in the later phase of the rewarming and remained at a low level during the first few days after rewarming. The following days the prothrombin and factor VII levels increased to levels well above the original ones. In the later phase of the rewarming the dogs showed a tendency to decrease of factor V in the two determinations made. During the days after rewarming the values tended to increase. The antithrombin activity of plasma showed a slight increase at the lowest temperature and shortly after rewarming there

was a marked rise which soon disappeared During the hypothermic state no significant changes in the fibrinogen content were registered but during the days after rewarming the fibrinogen level increased to higher values than the original ones During the cooling phase there was no fibrinolysis In the rewarming phase ▮ dogs showed increased fibrinolytic activity as judged by the Schneiders test In tests not reported in the table it was shown that the plasma taken from dogs during the hypothermic phase had a significant shortening effect on the recalcification time of human plasma

In the dogs kept at the lowest temperature for 2 to 6 hours there was no change in coagulation time recalcification time, prothrombin time or factor V content The prothrombin and factor VII showed a decreasing tendency The fibrinogen value was unchanged after 2 and 4 hours at 25 C but after 6 hours there was a decrease There were no signs of fibrinolysis during the hypothermic state but at the rewarming an increased fibrinolytic activity was noted The antithrombin activity increased at 2 and 4 hours but returned at 6 hours to the original value at 26 C At the same temperature the hematocrit increased from 42 per cent at 26 C to 46 5 per cent after 2 hours but then remained static Just after rewarming there was no difference between the values found in these dogs kept at a longer time at a low temperature and those cooled for a short period

Three dogs were cooled twice This material is small but a comparison reveals that the concentration of factor V in one dog and of fibrinogen in another were higher throughout in the second refrigeration experiment Furthermore there was a higher degree of fibrinolysis during the second refrigeration

Discussion

Our coagulation studies in hibernating and non hibernating hedgehogs showed that there was a prolongation of the coagulation time decrease of prothrombin and factor VII and an increase of antithrombin activity during the hibernation period The animals in series C from late August had also a low prothrombin content but no increased antithrombin activity A prolongation of the coagulation time and a reduction of the prothrombin content in hibernating hedgehogs as well as in hibernating bats hamsters and ground squirrels have been observed earlier (SUOMALAINEN and LEHTO 1952 SMITH LEWIS and SVIHLA 1954 SVIHLA BOWMAN and PEARSON 1952 RATHS and PERLICK 1953 SVIHLA *et al* 1951 SVIHLA BOWMAN and RITENOUR 1952) Our hedgehogs showed high factor V values during hibernation even when the changes in hematocrit are taken into consideration (BIORCK JOHANSSON and VEIGE 1956) This finding is contradictory to the results obtained by RATHS and PERLICK (1953) in hibernating hamsters

The present results concerning the increased antithrombin activity agree with those published by RATHS and PERLICK (1953) in hamsters and by SLOMA

LAINEN and his co-workers (1956) in hedgehogs. In our more detailed anti-thrombin studies it was shown that the activity of both antithrombin II and antithrombin III increased during hibernation. In addition we found that there was an increase also of the heparin co-factor activity of plasma. In this connection it should be remembered that the heparin co-factor exhibits species specificity as shown by MAGNUSSON and NILSSON (1956). RATHS and PERLICK and SUOMALAINEN *et al.* conclude from experiments with protamine sulphate titrations that the antithrombin is mainly heparin. SUOMALAINEN *et al.* have also shown an increased amount of mast cells in the hedgehog during hibernation (SUOMALAINEN and LEHTO 1952, SUOMALAINEN and HARMA 1951). On the other hand SMITH *et al.* (1954) could not obtain good correlation between mast cell counts of duodenal tissues and blood clotting times in summer or winter bats. We were not able to demonstrate any anticoagulant active in the first phase in the hibernating animal. As is well known heparin acts also as an inhibitor in this phase (NILSSON and WENCKERT 1954, DOUGLAS 1956 and SHANBERGE, SARELIS and REGAN 1959). Furthermore it was not possible to shorten the prolonged thrombin times and the recalcification times by addition of protamine chloride in various concentrations. Our results are thus not in agreement with those reported by RATHS and PERLICK (1953). In the interpretation of our observations it does not seem possible to explain the increased antithrombin titre as caused by free heparin trisulphuric acid. It is known that heparin from various sources and species differ in anticoagulant potency and mode of action (JAQUES, BELL and CHO 1954, MAGNUSSON and NILSSON 1957) that heparin may react with almost any protein. According to JORPES (1946) and JORPES and GARDELL (1948) differences in heparin activity may be ascribed to a varying degree of esterification with sulphuric acid and to the degree of polymerization. The possibility must be borne in mind that the increased antithrombin activity is caused by a small amount of heparin bound to proteins and or of low activity, for example heparin of low degree of esterification.

No earlier studies of the hedgehog fibrinolytic system could be traced. Our investigation showed no increased fibrinolytic activity. Thus the increased antithrombin activity in connection with the low prothrombin content would seem to be responsible for the slow coagulation in hedgehogs during hibernation and apparently is also sufficient to prevent intravascular coagulation.

Coagulation during hypothermia in man and different animals has been subjected to many studies but the findings are all rather equivocal.

Our studies on hypothermia in dogs which also included prolonged hypothermia during 2—6 hours showed only in a few dogs a tendency to shortening of the coagulation time and recalcification time during the hypothermic phase. During this period it was also found that the plasma caused a significant shortening of the recalcification time of human plasma indicating an increased thromboplastic activity. In parallel with this the values for prothrombin and

factor VII decreased and in some of the dogs a lowering of the fibrinogen and factor V levels were recorded At the end of the hypothermic phase and in the rewarming phase an increased antithrombin activity appeared During the hypothermic state there was no fibrinolysis but in the rewarming phase a moderately increased fibrinolytic activity could be observed in most of the dogs The days after hypothermia the values for prothrombin factor V and fibrinogen increased to values higher than the pretreatment levels of respective factors Many authors (WILLSON *et al* 1958, ROSS 1954 DETERLING *et al* 1955 TERZIOGLU and ÖZER 1956, BUNKER and GOLDSTEIN 1958) have noted a prolongation of the coagulation time FISHER *et al* (1955) and GRAY (1957) in their long term experiments with cooled dogs found that a prolongation of the clotting time soon developed FISHER *et al* (1955) write that by the fourth hour all animals demonstrated significant increase in coagulation time We kept dogs in a hypothermic state for as long as 6 hours without prolongation of their coagulation time ELLIS *et al* (1957) found no change in coagulation time when dogs were subjected to hypothermia alone but a rather pronounced shortening when the animals underwent circulatory occlusion and open cardiac surgery in the hypothermic state Our dogs underwent no surgery but nevertheless there was a shortening of the clotting time especially during the rewarming phase The varying results obtained by different authors are caused at least in part by differences in technique For example anesthetic methods have differed widely

Our results appear to be in agreement with those of ELLIS *et al* (1957) Like these authors we believe that the depletion of prothrombin factor V and fibrinogen is the result of intravascular clotting In our tests we were also able to demonstrate an increased thromboplastic activity This is similar to the process described by SCHNEIDER (1951) in patients with abruptio placentae In the same way as is the case for these patients we also found an increased fibrinolytic activity at the end of the hyper-coagulable phase A possible explanation might be increased cell destruction in the course of prolonged hypothermia resulting in a release of thromboplastic material and possibly also of fibrinolytic activators (ASTRUP) from the tissues to the circulating blood However we have not been able to demonstrate any histological changes in hypothermic animals (BJORKMAN HALL and JOHANSSON 1960

The changes in coagulation occurring in dogs during hypothermia appear to be induced by a mechanism other than that responsible for the reduced coagulation in hibernating hedgehogs

In an earlier study (BERGENTZ and NILSSON 1961) it was shown that when dogs were traumatized the fibrinolytic activity increased and the fibrinogen and factor V content decreased Administration of heparin prevented the decrease of these two last mentioned factors while the fibrinolytic activity remained unchanged It has been much discussed if the intravascular clotting or the fibrinolysis is the primary factor To decide this in hypothermic dogs the

experimental animals should be pretreated with heparin and ε amino caproic acid (wich inhibits plasminogen activation), respectively These studies are in progress

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The Relation Between Stimulus and Discharge in a Rapidly Adapting Touch Receptor

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Abstract

Lindblom U *The relation between stimulus and discharge in a rapidly adapting touch receptor* Acta physiol scand 1962 56 349—361 — A quantitative experimental analysis was made in decerebrate toads of the relation between stimulus and discharge in touch receptors of the common rapidly adapting type The stimuli consisted of mechanical pulses of variable slope amplitude and duration Recording was made from single afferent fibres in the dorsal root The impulse frequency of the discharge from the very rapidly adapting receptors and of the dynamic discharge from the less rapidly adapting receptors varied with the gradient of the stimulus in a characteristic way When the rate of the displacement was successively increased from the minimum effective value (critical slope) the impulse frequency was low at first and then rose as a logarithmic function of the displacement rate (Fig 2 3 5 and 6) The range of rates in which the change in frequency occurred was called the optimal slope range of the receptor The impulse frequency was independent of the amplitude of the displacement in the very rapidly adapting receptors (Fig 1 and 3 B) and only partially related to it in the less rapidly adapting ones The demonstrated relation between displacement rate and discharge frequency constitutes a basis for an exact reproduction of the temporal characteristics of the stimuli and may be of primary importance for tactile discrimination

In a recent investigation in which precise and graded mechanical stimuli were used it was demonstrated that the rapidly adapting touch receptors in the toad s skin are specifically excited by progressive deformation while steady

deformation is much less effective (HÖGLUND and LINDBLOM 1961) In the same investigation the rate of rise and the amplitude of the stimulus were also related quantitatively to the discharge The experiments in question were however, largely confined to determinations of critical slopes and slope threshold curves, i e to stimulation at threshold intensity Such determinations are of interest for the analysis of the excitability properties of the receptors but do not make it possible to deduce how stimuli of various types that may occur during natural life are reproduced in the afferent nerve Physiological stimuli will almost certainly represent a variety of gradients and amplitudes and may only incidentally be adjusted to the threshold values of the various receptors It was therefore considered to be of interest to analyse in the same type of preparation the response when these parameters were more widely varied This was made in the present investigation in which the impulse frequency and the duration of the discharge were registered at various supra threshold rates and amplitudes of displacement The results may be of general interest also in so far as they represent a systematic analysis of the relation between stimulus and discharge in a receptor that may be regarded as a prototype for a rapidly adapting sense organ

Methods

All experiments were performed on decerebrate and curarized toads (*Bufo bufo*) 26 touch receptors belonging to 23 sensory units with their receptive fields on the plantar surface of the hindfoot or on the lower leg were investigated more thoroughly Only touch receptors connected to coarse afferent fibres (A fibres cf. LINDBLOM 1958 p 29) i e of the rapidly adapting type were included The stimuli were applied vertically to the skin surface, the displacement of which was recorded simultaneously with the stimulation by means of a capacitance meter This procedure as well as the technique used for recording of the action potentials in single afferent nerve fibres has been described in detail earlier (LINDBLOM 1958) The mechanical stimulator was the same as used by HÖGLUND and LINDBLOM (1961) but its parameters had been modified so that more slowly rising stimuli could be obtained Thus the rate of rise could be varied continuously from 80 mm/sec down to 0 08 mm sec.

The maximal amplitude of the mechanical pulses was 300 μ This means that stimulus strengths amounting to several multiples of the rheobase could usually be obtained The rheobase i e the threshold amplitude for a single impulse on stimulation with a steep gradient was in most cases between 10 and 60 μ The maximum amplitude was also high enough to be representative of the degree of displacement that may occur during natural life The skin of the toad is soft and an adequate degree of deformation requires only a minimal force In regions such as the middle of the planta pedis and the lower leg where the subcutaneous tissue is loose the skin surface is readily displaced a mm or even more. On the digits, however the upper physiological limit for perpendicular displacements is lower due to the damping effect of the underlying skeleton, and may be only a few hundred μ

The mechanical stimulator was fed by two Grass stimulators. For amplification and recording the usual Grass preamplifier model P 6 and a Grass camera were used together with a Tektronix type 502 oscilloscope (AC-coupled

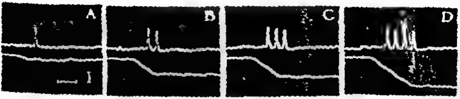

Fig 1 Discharges from single touch receptor (upper traces) elicited by stimulation with increasing amplitude but constant gradient (11.5 mm sec) Threshold for the first impulse 30 μ (A) Note the regular firing at constant intervals irrespective of the displacement amplitude in B C and D Lower traces (from capacitance meter) show the displacement of the skin surface Vertical bar in A 100 μ horizontal bar in A 10 msec

Results

By varying the gradient of the mechanical pulses while the amplitude was kept at a constant suprathreshold value and *vice versa* the influence of the two parameters of the stimulus could be studied separately to a satisfactory extent. The effect of varying the gradient was of greatest interest but in this context the amplitude will be considered first It may be pointed out that a certain minimum displacement was always necessary for discharge in these receptors Spontaneous firing was never seen during the present or earlier studies in which care was taken to keep the experimental conditions within physiological limits with respect to the blood circulation of the animal and the temperature and moisture of the skin Stimulation at high repetition rates which may lead to over stimulation and fatigue was avoided as well as stretching the skin prior to stimulation (cf LOEWENSTEIN 1956) It may also be mentioned that stimuli of subthreshold amplitude cannot summate to give a propagated response not even if they are applied simultaneously to two or more receptors supplied by branches from the same fibre (LINDBLOM 1958) Thus only threshold and suprathreshold displacements have to be considered as effective stimuli

The records in Fig 1 illustrate the typical result when a very rapidly adapting touch receptor was stimulated at a moderately steep gradient and the amplitude alone was varied As seen an increase of the stimulus amplitude from the threshold (record A was followed by a successive recruitment of new impulses (records B—D This was to be expected since the duration of the rising phase automatically increases with the amplitude and the effective part of the stimulus thus acts on the receptor for a progressively longer time What seems more interesting than the prolongation of the discharge however is that the impulse interval was remarkably constant which implies that the firing rate was independent of the amplitude of the displacement This is shown graphically for another similar receptor in Fig 3 B Thus, in the very rapidly adapting receptors, the amplitude of the stimulus influenced only the number of impulses per stimulus which was a linear function of the displacement in μ

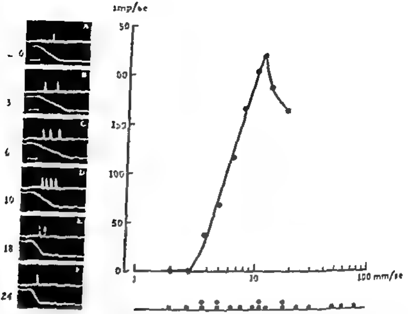

Fig 2 Records (A—F) showing series of responses obtained from a very rapidly adapting touch receptor by suprathreshold stimuli of different gradients indicated in mm/sec to the left of records. Horizontal bar in A 50 msec, in B 20 msec, in C 10 msec, same sweep speed in C—F. Plateau value of displacement in all records about 300 μ (rheobase 30 μ)

Diagram showing relation between displacement rate (abscissa, log scale) and discharge frequency (ordinate) in the same receptor. Frequency at 2.0 mm/sec (critical slope) and at 3.0 mm/sec where only one impulse was initiated plotted on zero line. Black dots below the diagram mark the number of impulses initiated at each rate

In the receptors in which the discharge was confined to the moving phase of the stimulus at moderate gradients, as in the receptors illustrated in Fig 1 and 2, the number of impulses per stimulus decreased when the gradient was made steeper. This was of course due to the fact that the effective part of the stimulus became shorter. Even if the displacement was allowed to proceed to its upper physiological limit (cf Methods), the rising phase might be so short that there was time for the initiation of only a few impulses. At least one impulse was always obtained with the steepest gradients used in the present series of experiments, about 80 μ/msec. In some receptors even such sharp stimuli produced a short repetitive discharge, and a few additional impulses that were initiated immediately after the end of the rising phase of the mechanical pulse was a rather frequent finding. When the effect of varying the stimulus amplitude was examined in these cases, the result was similar to that obtained on stimulation with lower gradients, i.e. the number of impulses of the discharge, but not the firing rate, was related to the plateau value of the displacement.

As pointed out by Höglund and Lindblom (1961), probably all touch receptors in the toad's skin connected to large afferent fibres (A fibres) are

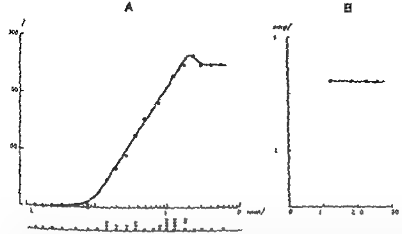

Fig 3 A Slope frequency curve from very rapidly adapting touch receptor with relatively wide optimal slope range Critical slope 0 14 mm/sec The number of impulses initiated at the various rates are denoted by black dots below the diagram B Amplitude frequency curve from same receptor obtained at a displacement rate of 13 mm/sec The threshold for the first impulse was 25 μ and for the second 110 μ

rapidly adapting in the sense that only discharges of limited duration are obtained on maintained mechanical stimulation with constant amplitude and reasonable force Among these receptors some are very rapidly adapting and produce only short bursts of impulses while others are less rapidly adapting and give longer bursts In the experiments in which the influence of the rate of the displacement was examined more closely these two variants of touch receptors showed differences in their behaviour so that they are best described separately

The *very rapidly adapting* receptors fired only during the phase of deformation following the onset of the mechanical stimulus and not at all during the plateau phase of the stimuli except on steep gradient stimulation in some cases as described above The minimum effective deformation rate (critical slope) was relatively high and the maximal number of impulses that could be initiated by a single stimulus was rather small Sometimes only a few impulses were elicited even if the amplitude of the displacement was many times the threshold value In Fig 2 records A—F show a series of responses obtained from a very rapidly adapting receptor at different deformation rates At a low rate as in A (2 mm/sec same as critical slope) or at a high rate as in F (24 mm/sec) only one impulse was initiated in spite of the amplitude of the displacement being about ten times the rheobasic value At intermediate rates (B—E) the response consisted of two to four impulses The firing rate plotted as frequency of impulses per second varied with the gradient of the stimulus in a characteristic way as is illustrated in the diagram in Fig 2 As seen the frequency

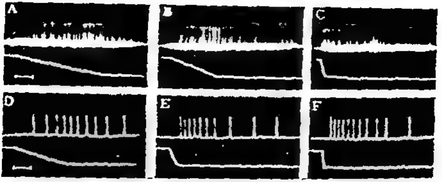

Fig 4 Responses from a less rapidly adapting touch receptor obtained at different rates of displacement. A—C same sweep speed time bar in A 200 msec. D—F same sweep speed, time bar in D 20 msec. Stimulus gradients in A 0 25 in B 0 5 in C and D 3.8 in E 24 in F 80 mm/sec. Plateau value of displacement same in all records about 250 μ

rose steeply and was linearly related to the logarithm of the gradient when this was increased from about 3 mm/sec to 11 5 mm/sec Above 11 5 mm/sec a moderate reduction of the frequency occurred (this reduction was usually slight cf. Fig 3 5 and 6) The frequency curve could not be drawn beyond 18 mm/sec since at still higher rates only one impulse was initiated in this receptor

The number of impulses per stimulus obtained at the various deformation has been denoted by filled circles on the line below the diagram in Fig 2 The maximum number of four impulses was initiated at 10 mm/sec, and at 11 5 mm/sec The latter gradient was the same at which the maximum frequency occurred In other receptors the maximum number of impulses was obtained over a wider range of rates Fig 3 A shows the slope frequency curve from a receptor in which the maximum of four impulses was elicited from 10 to 18 mm/sec This range corresponded to the upper part of the steeply rising section of the curve which means that the frequency of the discharge was also relatively high

A slope frequency curve of similar type as shown in Fig 2 and 3 A was obtained not only from the very rapidly adapting receptors but also as will be illustrated below from the less rapidly adapting ones The capacity of grading the intensity of the afferent discharge according to the gradient of the stimulus was thus a common property of all the rapidly adapting touch receptors The range of gradients where the impulse frequency varied with the displacement rate will be called the *optimal slope range* of the receptor Within this range the frequency was roughly proportional to the logarithm of the displacement rate In the very rapidly adapting receptors stimulation at rates in the optimal range also resulted in a greater number of impulses per stimulus than did stimulation at any other rate

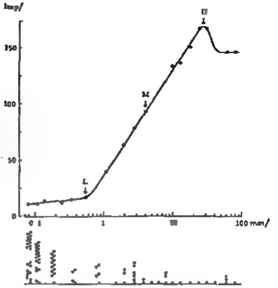

Fig 5 Slope frequency curve from less rapidly adapting receptor Critical slope 0.08 mm/sec L M and U mark the lower limit midpoint and upper limit resp of the optimal slope range Black dots below the diagram represent the number of impulses initiated at different rates of displacement. Note the increase in number at low rates

In contrast to the very rapidly adapting receptors the *less rapidly adapting units* discharged also during the plateau phase of the stimuli provided the amplitude of the displacement amounted to at least a few multiples of the rheobase The behaviour of the initial or dynamic discharge was similar to that of the very rapidly adapting receptors but certain differences were observed

The records in Fig 4 show the responses obtained from a less rapidly adapting receptor on stimulation at various deformation rates Typically the critical slope was comparatively low in this case less than 0.08 mm/sec (record A) which was the lowest rate of the stimulator As a consequence the duration of the rising phase was considerable at least for the first decade of rates above the critical slope (records A and B) The total number of impulses initiated during the rising phase was greatest at these low rates and underwent a successive reduction as the rate was increased This pattern was characteristic of the less rapidly adapting receptors Further the impulse interval was usually not constant but diminished towards the end of the rising phase at least at low deformation rates (Fig 4 A and B) The correlation between amplitude and firing rate was not close however At higher rates the impulses of the first part of the discharge were spaced more equally as in the discharge from the very rapidly adapting receptors or were closest at the beginning (Fig 4 E—F)

At low and intermediate displacement rates the dynamic discharge was considered as equivalent to the discharge initiated during the rising phase of the stimulus and could easily be separated from the plateau discharge because of the slow time relations There was also usually a pause or a sudden change in discharge frequency at the end

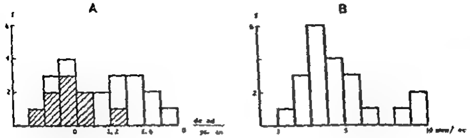

Fig 7 Histograms showing the distribution of the widths (A) and the midpoints (B) of the optimal slope ranges of 21 receptors. Stripping in A marks receptors classified as very rapidly adapting

Fig 5 also illustrates in black dots below the diagram the increase of the number of impulses per stimulus at low displacement rates For this particular receptor the number of impulses initiated at rates near the critical slope could not be determined since this was less than 0 02 mm/sec the lower limit of the stimulator Such a determination would probably have shown however, that the number of impulses decreases again near the critical slope This was indicated by the behaviour of a few receptors of intermediate type the critical slopes of which happened to be just within the range of the stimulator Fig 6 is from one of these receptors As appears from the representation in black dots below the diagram the total number of impulses, although having its maximum at low rates decreased successively as the critical slope was approached

In all the optimal slope range was determined for 21 receptors Nine of these were classified as very rapidly adapting nine as less rapidly adapting and three as intermediary The optimal ranges of the various receptors overlapped considerably and the whole sample appeared as a homogeneous group both with respect to the widths of the ranges and the absolute values of the range limits To illustrate this the distribution of the midpoints of the ranges was chosen The midpoint of the optimal slope range for example M in Fig 5, corresponds to half the maximum frequency of the receptor The abscissa at this point may be taken to indicate by a single value the sensitivity of the receptor with regard to its position on the scale of deformation rates As appears from the histogram in Fig 7 B all midpoints fell within one and the same decade of rates (1–10 mm/sec) The upper limits of the optimal ranges, i e the rates corresponding to U in Fig 5 showed a similar narrow distribution while the distribution of the lower limits (as L in Fig 5) was broader (about 1.5 decades A close comparison with the distribution of the critical slopes is not possible since for several receptors these were lower than the lowest rate of the stimulator and therefore remained hypothetical The available values indicate however that their distribution may have been over two decades

Discussion

The most conspicuous finding during the present investigation was the graded effect on the receptor discharge of varying the rate of rise of the stimulus. The fact that the rate of displacement of the skin surface could be signalled by means of the frequency code may be of primary physiological importance since it forms a basis for an exact reproduction of the temporal characteristics of the stimuli. The receptors concerned are connected to large afferent fibres with a relatively high conduction velocity, and adapt more or less rapidly. Further they are located superficially in the skin and have comparatively low thresholds. All these characteristics make the receptors suitable for tactile discrimination and the present results fit well with such a function.

The impulse frequency and to a certain extent, the number of impulses per stimulus were evidently the two most important parameters of the response that were influenced by the gradient of the stimulus. The latency and the rheobase are also influenced, mainly, however, at gradients just above the critical slope (HOGLUND and LINDBLOM 1961). As regards the rheobase, it is practically constant at all higher rates including those within the optimal range.

The discharge frequency was independent of the amplitude of the stimulus in the very rapidly adapting receptors, and at certain displacement rates also in the less rapidly adapting receptors. This finding was unexpected and may at first sight be surprising since from the more extensive knowledge of the behaviour of slowly adapting sense organs, one is used to think of the frequency as a function of the amplitude of the stimulus. In the rapidly adapting touch receptors however progressive deformation is relatively more important for the excitation than steady deformation. It is in analogy with the general principle of frequency modulation for signalling the strength of adequate stimuli to sense organs that the frequency of the discharge is related to the rate of the displacement instead of its absolute height in such receptors. The amplitude of a rising stimulus on the other hand is a function of the stimulation and determines the duration of the discharge.

The present experiments were concerned with the relation between skin displacement and impulse discharge and not directly with the various links in the transmission process such as the actual deformation of the mechanosensitive receptor structures and the formation of receptor and/or generator potentials. The interesting question of how these links may be interrelated to result in a regular relationship of the type found between the parameters of the stimulus and those of the discharge may however be discussed briefly.

The constant firing rate during the rising phase of the stimuli may suggest a direct relationship between the rate of displacement of the skin surface and the height of the excitatory state in the sensory ending which initiates the action potentials. There is evidence however that the linkage is considerably more complex. Refractoriness of the nerve endings e g comes in as a factor

which is relevant for the formation of the discharge. The impulse frequency of the latter may ultimately be regarded as the outcome of a balance between excitation and refractoriness. The refractory state is long lasting in these receptors and shows summation even at low repetition rates (LINDBLOM 1958). It therefore influences the excitability at all stimulus gradients yielding repetitive discharges. The summation curve is not linear (LINDBLOM 1958, Fig. 20) and has to be matched with a non linear excitatory increase to result in the regular discharge actually recorded. It is therefore not to be expected that the relations between the individual links in the receptor mechanism should be simple linear ones. This at least does not seem to be the case for the last stage, which is supposed to be the transition from a generator potential to action potentials. By stimulating the endings directly with slowly rising currents as a model experiment it might be possible to show which wave form a generator potential should have to initiate a regular discharge in these receptors (cf. GRANIT and SKOGLUND 1943 and KATSUKI and YOSHINO 1952).

Certain conclusions can be drawn from the results presented concerning the integration in the sensory units to which the receptors belong. The nerve fibres quite often branch peripherally and innervate several receptors. When more than one receptor belonging to the same unit are activated, the receptor that is capable of initiating the highest impulse frequency alone is responsible for the discharge in the main fibre, while the other endings are blocked (LINDBLOM 1958). The consequence of this interaction can now be related directly to the characteristics of the stimulus as follows. If stimulation proceeds at different gradients in the same receptive field, the unit at each moment picks up the stimulus, or the part of the stimulus, which has the steepest gradient. Discrimination will of course not occur between two gradients that both are outside the optimal slope range of the receptors. Further, a prerequisite is that the various receptors of a particular unit are about equally sensitive to the gradient. Preliminary experiments performed during the present investigation indicate that this may be the case.

It would no doubt be of great interest to know if the results obtained on the toad also apply to touch receptors of higher animals. So far detailed data on the relation between suprathreshold stimuli and discharge are not available for any other species. It seems conceivable, however, that not only various touch receptors but also other types of rapidly adapting mechanoreceptors may behave similarly. As regards e.g. the movement sensitive joint receptors in the shore crab *Carcinus maenas* BURKE (1954) found that the discharge frequency increased with the velocity of the stimulus. Qualitatively parallels may also be drawn with slowly adapting mechanoreceptors capable of giving phasic responses to rapid changes of the stimulus. In muscle spindles, e.g., it is known that the frequency of the dynamic discharge initiated on the application of stretch is dependent on the rate of rise of the stimulus (MATTHEWS 1931, Fig. 8; MATTHEWS 1933, Fig. 3; KATZ 1950). The same phenomenon

has been observed for pressure receptors (ADRIAN and ZOTTERMAN 1926 Fig 8) and certain joint receptors in the cat (ADRIAN and UMRATH 1929 p 142, BOYD and ROBERTS 1953 Fig 7 SKOGLUND 1956 Fig 17)

Certain differences were observed between the discharge pattern of the very rapidly adapting receptors and that of the less rapidly adapting ones From a functional point of view it seems more relevant to emphasize the similarities however The effect of varying the parameters of the stimuli indicated that the dynamic discharge in the less rapidly adapting receptors was equivalent to the discharge in the very rapidly adapting ones Further, the optimal slope ranges of the various receptors overlapped considerably Neither the distribution of the ranges along the scale of deformation rates, nor that of their widths (Fig 7) indicated a separation into subgroups (*cf* HÖGLUND and LINDBLOM 1961, p 118) It may be more accurate, therefore, to consider the very rapidly adapting and the less rapidly adapting touch receptors as variants within a population than as separate types of receptors This view seems to be supported by the results of recent subthreshold excitability studies which have shown that a static excitatory change may occur *also in the very rapidly adapting receptors* (LINDBLOM 1963)

The repetitive firing obtained in some of the very rapidly adapting receptors on steep gradient stimulation indicates that the dynamic excitation may outlast the period of progressive deformation and remain strong enough to produce a discharge early in the plateau phase before it subsides A comparison may be drawn with the receptor potential in the Pacinian corpuscles in the cat s mesentery which are as rapidly adapting as the most rapidly adapting touch receptors in the toad skin In consistency with the rapid adaptation receptor potential declines fast during steady stimulation but its time con is long enough to allow a duration of several msec following the appli on of a rectangular mechanical pulse (GRAY and SATO 1953) In the ptors studied here some prolongation of the discharge into the plateau se of the stimulus may also be explained by summation of residual dynamic excitation and subthreshold static excitation produced by maintained displacement (LINDBLOM 1963) If it is assumed that impulses can be initiated only during progressive deformation the repetitive firing on steep gradient stimulation would imply that the deformation in the mechano-sensitive receptor structures may be considerably slower than the surface displacement visualized on the stimulus record This is not likely, however, since there is evidence that the inertia of the tissue is slight (*cf* HÖGLUND and LINDBLOM 1961 p 117)

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Electrophysiological Investigation of the Gustatory Effect of Various Biological Sugars

By

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Abstract

ANDERSEN, H. T., M. FUNAKOSHI and Y. ZOTTERMAN. *Electrophysiological investigation of the gustatory effect of various biological sugars.* Acta physiol. scand. 1962. 56. 362—375. — The stimulation characteristics of various sugars found in biological material have been studied in the mongrel dog. Equimolar solutions of D-fructose, L-sorbose, D-galactose, D-glucose, D-mannose, sucrose, maltose and lactose were applied to the tongue of the experimental animal, and the electrical response in the whole chorda tympani or in functional single fibres of this nerve recorded and analyzed. The following order of decreasing stimulating ability have been established for the sugars tested using the electrical response recorded from the un-split chorda tympani as a basis for comparison: D-fructose > sucrose > L-sorbose > D-mannose > D-glucose > maltose > D-galactose > lactose. The information obtained from functional single fibres generally verified this order of the various sugars, with the exception that maltose in a few instances elicited an unusually large response. A comparison of the physical constants of the sugars revealed that large stimulating ability of a sugar appeared to be in some way related to high water solubility of the molecule. All of the fibres responding to the sugar solutions were tested with solutions of sodium chloride and quinine. Some fibres appeared specific for sweet stimuli whereas others responded to sodium chloride as well. Response to sugar and quinine was never observed in the same fibre. The results obtained by this direct method of study agree well with earlier psychophysiological investigations.

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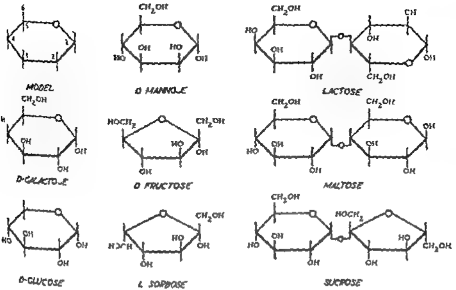


Fig 1 Structural formulas of sugars investigated

Sweet taste is elicited in humans by a wide variety of substances but is generally associated with the sugars

Besides being of great chemical interest the sugars constitute the most important group among the sweet tasting compounds because of their nutritional value as well as for economical reasons

The chemistry and physics of the sugars have been studied intensively and some detailed information about the molecular configurations involved is available especially concerning the sugars found in biological material

In spite of their closely related molecular structure the sweet stimulating ability of the various sugars differs markedly (Becker and Herzog 1906 Biester Wood and Wahlin 1925 Fabian and Blum 1943) The following order of decreasing sweetness has been quoted for a series of common sugars in a recent review (Pfaffmann 1959) Fructose > sucrose > maltose > glucose > lactose

Even the α and β configuration of the same sugar may have different stimulating characteristics Thus Cameron (1947) reported that a freshly prepared solution of α D glucose tasted sweeter than a solution 10—16 hours old in which mutarotation had taken place Pangborn and Gee (1961) have repeated the experiment and explored this characteristic in several other sugars They found that the α configuration of D fructose D glucose and D galactose are sweeter than the β forms whereas β lactose is sweeter than α lactose

Not all sugars are sweet however One of us (H T A) investigated the gustatory sensations elicited by the three stereo isomeric aldohexoses D galactose,

Table I[1] *Phys cal properties of various mono and disaccharides*

Sugar	Formula	Molecular weight	Density g/ml	Melting point	Water solubility g/100 ml 17 5 C	Water solubility g/100 ml 35 C	Natural sources
D-fructose	$C\,H_{1}\,O_6$	180 16	$1{,}598\frac{25}{4}$	104	very soluble		Seminal fluid
L-sorbose	$C_6H_{1}\,O$	180 16	—	—	—	—	Made from D sorbitol by conversion by *A etobacter suboxyda s*
D-galactose	$C_6H_{12}O$	180 16	—	168	52	—	Component of lactose. Free in mammals with congenital galactemia
D-glucose	$C\,H_1\,O_6$	180 16	$1\,544\frac{25}{4}$	146	83	—	All higher animal and plant organisms
D-mannose	$C\,H_1\,O$	180 16	$1{,}539\frac{20}{4}$	132	248	—	*Constituent of polysaccharides and glycoproteins*
Lactose	$C_1\,H\,{}_1O_{11}\,H\,O$	60.31	$1{,}525\frac{20}{4}$	—	15	27	Milk
Maltose	$C_{12}H\;O_1\;H\,O$	360 21	$1{,}540\frac{20}{4}$	—	76	101	Degradation of starch and other polysaccharides
se	$C_{12}H\;O_{11}$	342 30	$1\,588\frac{15}{4}$	—	200	230	All photosynthetic plants

[1]After Handbook of Chemistry and Physics. Thirty-seventh Ed. *Chemical Rubber Publishing Co* Cleveland 1955 A. SEIDELL Solubilities of *Organic* Compounds. *D van Nostrand Company Inc* New York 1941 J S. FRUTON and S SIMMONDS General Biochemistry Second Ed. *John Wiley & Sons Inc* New York 1959

D mannose and D-glucose in 15 human subjects and found that whereas the D-galactose and D glucose produced varying degrees of sweet sensations D mannose was by the subjects reported to taste bitter The threshold value for D-mannose was also found to be significantly lower than those of D galactose and D-glucose Recently STEINHARDT CALVIN and DODD (1962) have demonstrated that α D mannose is sweet while the mixture of α D mannose and β-D mannose is bitter

All of these previous investigations have been carried out as discrimination tests in human subjects The purpose of the present investigation was to obtain

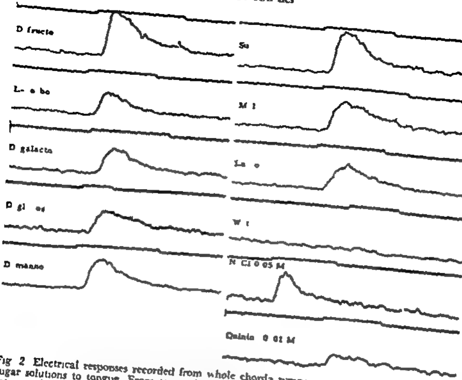


Fig 2 Electrical responses recorded from whole chorda tympani upon application of 0 5 M ugar solutions to tongue From top to bottom are reco ded gnal f om dispensing burette integrated response and time marks T me in sec

direct quantitative information as to the different stimulating properties of various sugars found in biological material using electrophysiologic technique

Previous electrophysiological studies have revealed that the dog possesses specific sweet fibres in the afferent nerves from the tongue (ANDERSSON *et al* 1950) thus animal was therefore chosen to serve as subject in the present investigation

Material and methods

Eight mongrel dogs of both sexes were used in the present study Their body weights ranged from 5 0 to 9 0 kg They were anesthetized with a 6 solution of Mebumal natrium one ml of which contains 18 mg mebumal 40 mg mebumalsodium and 250 mg urethane An initial dose of 0 6 ml kg body weight of this solution was given intravenously Ad I tional doses of 0 5—1 0 ml of the solut on diluted with Ringer s solution to 10 ml were injected whenever needed through a venous cannula

The taste fibres running in the chorda tympani were studied since all four classical taste qualities as well as specific water sensitivity are known to be present in this structure in the dog (APPELBERG 1958

The operative procedure was performed accord ng to the description of ZOTTERMAN (1936 with the mod ficat ons and precautions recommended by GORDON *et al* (19

Table II Relative sweetness or stimulation ability of sugars

Sugar	BECKER and HERZOG	BIESTER WOOD and WAHLIN[1]	FABIAN and BLUM	WALTON	ANDERSEN FUNAKOSHI and ZOTTERMAN
D fructose	2	173 (1)	1	103—150 (1)	120 (1)
L-sorbose	—	—	—	—	[illegible] (3)
D galactose	6	37 (4)	—	—	59 (7)
D glucose	3	74 (3)	3	50— 60 (3)	70 (5)
D-mannose	—	—	—	—	77 (4)
Lactose	5	16 (6)	5	27— 28 (5)	53 (8)
Maltose	3	32 (4)	4	60 (3)	67 (6)
Sucrose	1	100 (2)	2	100 (2)	100 (2)

[1] Equal weights of the sugars used in the test solutions

The electrical response from the whole chorda tympani nerve was recorded by means of an electronic integrating device before it was split up into fine strands containing a few or only one functional single fibre The electronic apparatus and the experimental technique employed have been described in many previous papers from this laboratory (LILJESTRAND and ZOTTERMAN 1956 KITCHELL *et al* 1958 GORDON *et al* 1959 and KONISHI and ZOTTERMAN 1961) and will therefore not be repeated here

Eight sugars 5 monosaccharides and 3 disaccharides *all of them* found in biological material were investigated for their stimulating ability They included three stereoisomeric aldohexoses D galactose D glucose and D mannose two ketohexoses D fructose and L-sorbose and three disaccharides lactose maltose and sucrose All sugar solutions were of 0 5 M strength so that the taste receptors of the tongue were always stim[ulated] by the same number of molecules The solutions were made up from analytical [grade] supplied by Kemoaktiebolaget KEBO AB Stockholm Table I and Fig 1 [give] additional information on the physical and chemical properties of the sugars [The fib]res responding to the sugar solutions were also tested with water a 0 5 M solution of sodium chloride and a 0 01 M solution of quinine hydrochloride

Fresh solutions of all sugars were prepared 2—3 hours prior to use Thus ample time was left for mutarotation to take place until equilibrium was reached and simultaneously fermentation was kept minimal

Results

Integrated responses

The integrated response of the whole chorda tympani nerve was recorded in all of the dogs In Fig 2 the results obtained in one experiment are reproduced

By a suitable arrangement of the dispensing apparatus it was possible to avoid appreciable stimulation of the mechanoreceptors when the different solutions made contact with the surface of the tongue Water alone did not produce any detectable response in this preparation All of the sugar solutions however produced conspicuous bursts of electrical activity in the chorda tympani when

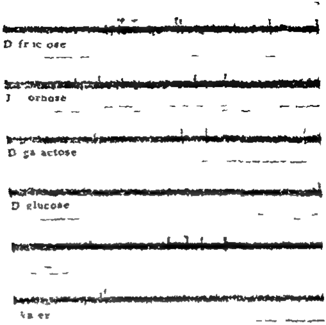


Fig. 3. Records obtained from small strand of chorda tympani containing only one functional fibre. Time in sec.

applied to the tongue. D-fructose invariably proved to be the strongest stimulus among the sugars, next followed by sucrose. Among the aldohexoses D-mannose almost always elicited the largest response. D-galactose and D-glucose tended to vary somewhat from one experiment to another as to which one would produce the greater effect. Thus in the exp. shown in Fig. 2 D-galactose turned out to evoke the larger response, but on the whole D-glucose appeared to be the most potent stimulus of the two. L-sorbose was frequently found to be approximately half as strong a stimulus as D-fructose.

The response pattern resulting from stimulation with the three disaccharides was very clear cut, and established in every experiment the following order of decreasing stimulating ability: Sucrose > maltose > lactose.

An average value for the sweetness of each sugar is presented in Table II. These figures have been arrived at by using all experimental data obtained from the whole chorda tympani by means of the integrator. The deflection from the base line produced by sucrose has been given an arbitrary value of 100 units in each individual experiment. The relative sweetness of the other sugars have been determined on this basis, and the corresponding means for the whole series of experiments calculated. The reason for using sucrose as a standard with a value of 100 units is to make our results comparable to those of previous workers. The results obtained by Becker and Herzog (1906), Biester, Wood and Wahlin (1925) and Fabian and Blum (1943) are provided in Table

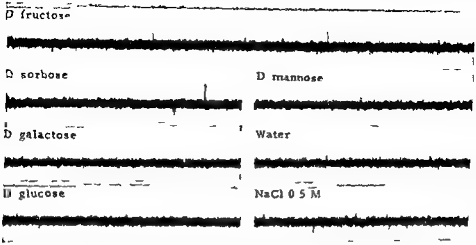


Fig 4 Functional single fibre from the chorda tympani responding to D fructose only among the 0 5 M solutions of monosaccharides tested Time in sec

for comparison Where no quantitation has been attempted by the authors the sweetest sugar has been identified with no 1 the next with no 2 and so on

The integrated responses from the whole chorda tympani nerve were also recorded when the tongue was stimulated with salt (0 5 M NaCl) and bitter (0 01 M quinine hydrochloride) and are shown in Fig 2 The salt solution produced a relatively small and compared with the sugars rather phasic response The bitter stimulus elicited only a very slight effect

The magnitude of the integrated response is determined not only by the er of active fibres and their frequencies but also by the size of the in l spike potentials Hence although of great statistical interest the in ated response can only be regarded as a relatively crude estimate of the g potential of the various sugars Therefore the question whether ructose and sucrose elicit their large responses by activating more fibres than the other sugars or by stimulating the same receptors with a greater frequency can only be answered through the study of the responses from functional single fibres

Individual fibres Monosaccharides

Figs 3 and 4 present the responses produced in two different functional single fibres after stimulation of the tongue with monosaccharides The fibre shown in Fig 3 exhibited some spontaneous discharge and was stimulated by all of the five sugars D fructose appeared to be more than twice as effective as D mannose and L-sorbose which were equally potent stimuli The activity of the fibre resulting from stimulation with D glucose was only half of that of D mannose and L-sorbose but twice as large as that of D galactose which in

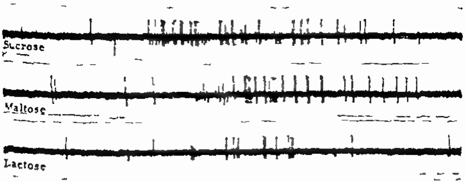


Fig 5 Electrical responses recorded from functional single fibre upon application of 0 5 M solutions of disaccharides to the tongue Time in sec

this case elicited the smallest response This fibre did not respond to water, salt or quinine

Fig 4 shows a fibre which did not display spontaneous discharge Among the sweet stimuli only D fructose elicited electrical activity in this preparation but like the fibre in Fig 3 no response was produced when water 0 5 M NaCl or 0 01 M quinine hydrochloride were applied This type of fibre which only responded to the strongest stimulus among the 0 5 M sugars was fairly frequently observed

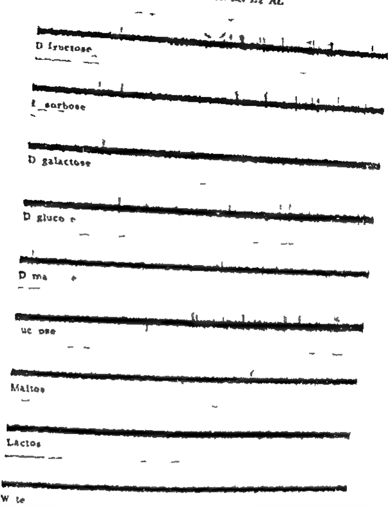
Disaccharides

The responses of a functional single fibre to the application of 0 5 M solutions of the disaccharides to the tongue of the dog is reproduced in Fig 5 The activity in this fibre resulting from such stimulation confirm the findings obtained by means of the integrator namely that sucrose elicits the largest response of the three and lactose the smallest maltose being the middlemost In this case maltose evoked approximately half as many spikes per unit time as sucrose did and twice as many as lactose

It may also be observed that the time elapsing from the stimulus reached the tongue until the response could be recorded was shorter in the case of sucrose than for the two other sugars Spontaneous discharge was exhibited by this fibre but quinine salt and water failed to produce any effect

Complete sugar series

The various responses produced in a fibre by all of the different sugars are shown in Fig 6 These follow roughly the same patterns as described in the section on the integrated responses D fructose was as usual by far the most effective stimulus in terms of spikes recorded per unit time while the response to sucrose was about 2/3 that of D fructose By the same means of comparison D galactose D glucose L-sorbose and maltose elicited very nearly the same



.. 6 Records from thin strand of chorda tympani containing only one functi nal fibre Note the large responses to D-fructose and sucrose as compared to those of D galact se and lactose Time in sec

response all of them being approximately half as effective as sucrose The smallest responses seen in thi fibre were produced by lactose and somewhat surprisingly D mannose Both of the latter were only half as strong stimuli as the group previously discussed

Unspecific fibres

The functional single fibres so far described responded to sweet stimuli only We found however some fibres which responded to a 0 5 M sodium chloride solution as well as to the sugars An example is furnished in Fig 7 Among the 0 5 M sugar solutions used only D fructose and sucrose were able to produce a clear cut response in this fibre Thus as far as stimul tion with monosac

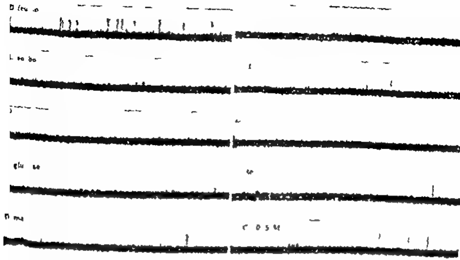


Fig 7 Records sho ving an unspecific functional single fibre from the cho da tympani This fibre respondeu to salt as well as to sugars Time n sec

charides is concerned this fibre has a certain resemblance to the fibre shown in Fig 4 Water did not elicit any electrical activity in this preparation but it responded to 0 5 M NaCl with exactly as many spikes per unit time as it did to sucrose

"Maltose fibres

Finally the peculiar stimulating properties of the disaccharide maltose ought to be mentioned

In a few preparations it was observed that this particular sugar elicited a massive volley of impulses upon stimulation whereas the other sugars caused a much smaller response Furthermore the magnitude of the responses of the other sugars did not differ very much in preparations in which the conspicuous response to maltose was observed

Unfortunately it has not been possible to obtain a very good single maltose fibre , but in Fig 8 a record obtained from a very thin strand of the chorda tympani containing two functional fibres is provided The unit displaying smaller spikes is probably a salt fibre primarily judging from the response to a 0 5 M solution of NaCl but it is also to some extent stimulated by some of the sugars The larger unit did not respond appreciably to salt whereas all of the sugars produced a definite increase in its rate of firing The most peculiar feature of this fibre is however its vigorous response to stimulation with maltose It has therefore tentatively been classified as a maltose fibre The same phenomenon has been observed in other preparations (Table III fibres 2 11)

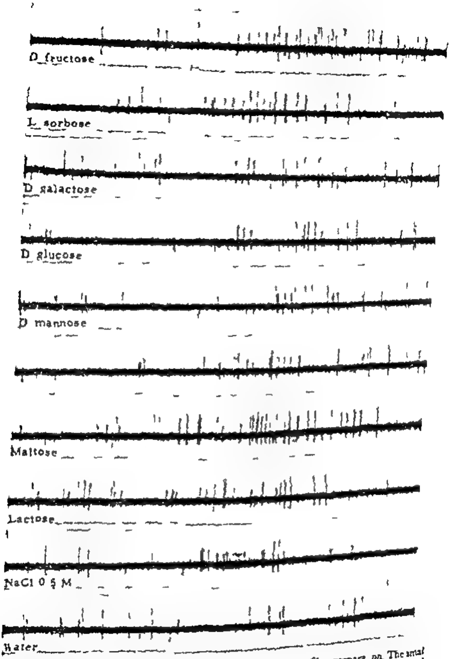


Fig. 8. Maltose fibre. Records show electrical responses in a two-fibre preparation. The small spike shows an unspecific salt sugar fibre. The larger spike appeared after stimulation with 0.5 M sugar solutions but especially after application of maltose. Time in sec.

Table III Characteristics of all functional single fibres studied

Numerator: Number of spikes Denominator: Time in sec

Fibre no.	D fructose	L-sorbose	D-galactose	D glucose	D-mannose	Lactose	Maltose	Sucrose
1	13/10	0/10	0/10	0/10	0/10	—	—	—
2	60/3	80/3	51/3	29/3	80/3	51/3	93/3	43/3
3	151/5	35/5	38/5	37/5	59/5	72/5	75/5	85/5
4	99/5	38/5	13/5	22/5	40/5	—	—	—
5	103/5	31/5	35/5	30/5	18/5	15/5	37/5	70/5
6	63/5	—	—	—	—	19/5	39/5	63/5
7	39/4	1/4	1/4	2/4	4/4	3/4	4/4	19/4
8	89/5	27/5	19/5	12/5	28/5	18/5	45/5	97/5
9	24/5	30/5	1[illegible]/5	20/5	32/5	27/5	5/5	15/5
10	44/5	16/5	8/5	18/5	14/5	11/5	7/5	16/5
11	53/5	44/5	45/5	35/5	32/5	41/5	74/5	45/5

Another characteristic of the preparation reproduced in Fig 8 is that whereas the sweet stimuli primarily elicited a response from the larger unit and to a lesser degree also activated the smaller the salt solution produced a phasic burst of impulses in the smaller one but inhibited the activity in the larger

The characteristics of all single fibres studied in the present investigation are summarized in Table III Here their impulse activity in response to different stimuli used have been provided in terms of number of impulses during a certain measured time

Discussion

The results obtained in previous psychophysiological studies and our electrophysiological findings (Table II) establish essentially the same rating for the various sugars as to their relative sweetness or stimulating ability respectively The electrophysiological evidence, therefore appears to provide a quantitative confirmation of the earlier investigations

Whereas the over all picture presents itself very much the same by these two methods of study the investigations of the functional single fibres described have furnished some new detailed information

Thus some of the functional single fibres studied were activated by all of the sugars tested (Fig 6 Table III) All of these fibres exhibited the largest response to D fructose and then next to sucrose We also observed some fibres which displayed electrical activity only after stimulation of the tongue with these two sugars remaining silent after the application of all of the other sweet stimuli (Fig 4 and 7) This seems to indicate that sweet receptors with different threshold values exist so that D fructose and sucrose elicit their

responses from the whole chorda tympani nerve not only by setting up higher frequency in the individual fibres than that of the other sugars but also by activating more fibres

The conspicuous differences in stimulating ability displayed by the various sugars must be explained by their different chemical or physical properties and by receptor characteristics

The molecular structure of the sugars are shown in Fig 1 D fructose appears in this figure in the furanose form, in which it participates in the formation of sucrose It should be remembered however, that in a solution of free D fructose the pyranose form dominates (FRUTON and SIMMONDS 1959 p 400)

One very interesting observation may be made by comparing the molecular structures of the sugars (Fig 1) and their stimulating power (Table II) It will appear that among the monosaccharides the magnitude of the response follows the order D fructose > D glucose > D galactose And similarly, for the disaccharides sucrose > maltose > lactose In all of these three disaccharides D glucose constitutes one half of the molecule, the other participant being D fructose, D glucose and D galactose respectively Hence the following rule appears established for the six sugars involved in this consideration Since all of the disaccharides have D glucose in common as one half of the molecule their relative sweetness and stimulating ability appears determined by the other constituent monosaccharide in the molecule Furthermore each disaccharide is less effective than an equimolar solution of that monosaccharide participating in its molecule which is not common to the other two disaccharides or D fructose > sucrose > D glucose > maltose > D galactose > > lactose These results seem to prove that the disaccharide molecules are not split when the solutions of sucrose maltose or lactose are applied to the tongue of the dog If namely an appreciable cleavage of these molecules into the constituent monosaccharides had taken place, the actual number of stimulating sugar molecules would have been very much increased over that of the solutions made up from pure monosaccharides Thus the solutions of sucrose maltose and lactose would have been expected to elicit a correspondingly larger response

At least one of the physical properties supplied in Table I fits perfectly with this rule namely the water solubility The conclusion as far as the sugars are concerned must therefore be that greater water solubility is associated with sweeter taste and stronger stimulating ability This of course does not necessarily imply that the latter properties depend directly on the water solubility It may as well indicate that the molecular characteristics responsible for water solubility also are important as to sweet stimulation

Finally, there is no parameter which correlates obviously with the massive stimulation sometimes produced by maltose (Table III fibres 2 11) and the interpretation of this phenomenon remains obscure

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Acta physiol scand 1962 56 376—380

From the Department of Physiology Karolinska Institutet Stockholm 60 Swed

Effect of Reserpine and Hypogastric Denervation on the Noradrenaline Content of the Vas Deferens and the Seminal Vesicle of the Guinea-Pig

By

Nils O Sjöstrand

Received 28 May 1962

Abstract

Sjöstrand N O *Effect of reserpine and hypogastric denervation on the noradrenaline content of the vas deferens and the seminal vesicle of the guinea pig* Acta physiol scand 1962 56 376—380 — The vas deferens and the seminal vesicle of the guinea pig contained the high noradrenaline concentrations of 10 ± 0.56 and 4.3 ± 0.26 µg/g tissue respectively but negligible amounts of adrenaline After reserpinization no measurable amounts of noradrenaline remained in the organs Following partial removal of the hypogastric nerve the noradrenaline content of the organs was only slightly reduced to 8 ± 0.76 and 4.2 ± 0.56 µg/g tissue respectively The probable existence of a peripheral synaptic mechanism in the sympathetic innervation of the vas deferens and the seminal vesicle is discussed

In a previous report (Sjöstrand 1962) it was shown that ganglionic blocking agents inhibit the motor response of the guinea pig vas deferens to hypogastric nerve stimulation These results indicate that a peripheral (cholinergic) synaptic mechanism is involved in the sympathetic innervation of the guinea pig vas deferens If this is the case then section and degeneration of the hypogastric nerve branches to this organ should not be accompanied by the reduction in catecholamine content which is seen in other organs after postsynaptic sympathetic denervation (Euler and Purkhold 1951)

The present study was undertaken in order to determine the effects of reserpinization and hypogastric denervation on the catecholamine content of the ductus deferens and the seminal vesicle of the guinea pig

Table I Effect of hypogastric denervation on the noradrenaline content of the vas deferens and the seminal vesicle of the guinea pig

Guinea pig no	Body weight (g)	Weight of pair of Vasa deferentia (g)	Noradrenaline in vas deferens (µg/g tissue)	Weight of pair of seminal vesicles (g)	Noradrenaline in seminal vesicles (µg/g tissue)
Controls					
1	380	0.11	10.0	0.42	5.2
2	610	0.16	9.7	0.94	5.1
3	485	0.16	11.2	0.65	4.0
4	570	0.18	8.7	0.86	3.6
5	450	0.10	11.8	0.49	4.1
6	560	0.16	8.3	0.63	3.9
Mean ± S.E.	501 ± 33	0.14 ± 0.02	10.0 ± 0.56	0.67 ± 0.08	4.3 ± 0.26
Denervated					
7	395	0.10	6.8	0.50	6.0
8	500	0.16	6.4	0.88	3.3
9	455	0.12	7.4	0.46	4.4
10	515	0.13	10.5	0.55	4.4
11	550	0.15	8.9	0.84	2.8
Mean ± S.E.	483 ± 27	0.13 ± 0.01	8.0 ± 0.76	0.65 ± 0.09	4.2 ± 0.55
Difference controls–denervated	—	—	2.0 ± 0.9 (0.1 > p > 0.05)	—	0.1 ± 0.58

Earlier studies have indicated the presence of relatively large amounts of adrenaline like material in the vesicular gland and ampulla ductus deferentis of various species (Euler 1934) and more recently noradrenaline has been found in large amounts in the vesicular gland of the steer (Euler 1961)

Material and methods

Sixteen guinea pigs weighing about 400—600 g were divided in 3 groups One group of 6 animals served as controls and received no treatment for the 10 days the experiment lasted One group of 5 animals received no treatment the first 8 days of the experiment. The animals were injected with 0.5 mg/kg reserpine (Serpasil®) subcutaneously 48 hours before the end of the experiment and 24 hours later they were given 1.5 mg kg reserpine by intraperitoneal injection One group of 5 animals was submitted to hypogastric denervation during nembutal anesthesia supplemented when necessary with ether The abdomen was opened with a midline incision The hypogastric nerves were removed from their origin below the kidneys to a point close to the seminal vesicles After removal of about 5 cm of the hypogastric nerve on each

side the abdomen was closed. The animals were sacrificed 10 days after the operation by a blow on the head. The ductus deferens and the seminal vesicle were removed and cleaned and their contents of secretions and spermatozoa were squeezed out as much as possible. The denervation was checked by electric stimulation in the area where the hypogastric nerve is normally located: in no case was a contraction of the vas deferens or the seminal vesicle seen.

After removal the organs were weighed and each pair of organs was homogenized with an Ultra Turrax apparatus in 20 ml of 5 per cent trichloroacetic acid. After 30 min extraction the organ extracts were filtered and the catechol amines were adsorbed on columns of alumina, eluted and estimated according to the method of EULER and LISHAJKO (1961). Recovery experiments yielded 88—99 per cent of added amounts of noradrenaline, the mean of 6 experiments being 95 per cent.

Results

Table I gives the individual data obtained from the control group and the denervated group, the mean ± S. E. of each group and the difference in noradrenaline content between controls and denervated animals.

As seen from Table I there is a slight reduction in noradrenaline content following removal of the hypogastric nerves. The difference in noradrenaline content between control animals and denervated animals is, however, not statistically significant by the Student T test and in the case of the seminal vesicle is absolutely negligible. There are no significant differences between the two groups in other respects, e. g. in body weights and weights of the vasa deferentia and the seminal vesicles.

No measurable amounts of noradrenaline were found in the vasa deferentia and the seminal vesicles from the reserpine treated group.

If present at all, adrenaline in the controls and in the denervated specimens constituted less than 5 per cent of the total catechol amines.

Discussion

It has been shown in the present study that the vas deferens and the seminal vesicle of the guinea pig contain high amounts of noradrenaline. Reserpine depletes the noradrenaline stores of these organs, but they are not overtly affected by partial removal of the hypogastric nerve.

The motor innervation of the vas deferens and the seminal vesicle seems to be exclusively sympathetic (LANGLEY and ANDERSON 1896) via the hypogastric nerve. The transmitter released on hypogastric stimulation is probably adrenergic (HUKOVIC 1961, SJÖSTRAND 1962 to be published). The motor response of the guinea pig vas deferens to hypogastric nerve stimulation is, however, blocked by ganglionic blocking agents (SJÖSTRAND 1962) indicating a peripheral synaptic mechanism, presumably cholinergic, which the nerve stimulus has to pass before it releases the adrenergic transmitter. The noradrenaline content of peripheral organs is greatly reduced by postsynaptic

denervation (EULER and PURKHOLD 1951, GOODALL 1951) but not by preganglionic denervation (REHN 1958). The fact that in the present report hypogastric denervation caused little or no decrease in the amount of noradrenaline in the vas deferens and the seminal vesicle therefore strongly indicates the presence of a peripheral synapse. Another explanation of the present results might be that the noradrenaline content of the vas deferens and the seminal vesicle is derived chiefly from other sources than the hypogastric nerve. This explanation lacks, however, morphological and physiological support and would require an abundant sympathetic innervation of these organs not serving motor purposes. The small insignificant reduction in noradrenaline content of the vas deferens observed after removal of the hypogastric nerve (if not due to random effects) might be explained by some fibers deriving from synapses located in the removed part of the hypogastric nerve or above it. In this respect the findings of BURNSTOCK and HOLMAN (1962) are of particular interest. They found that 8—13 days after nerve section stimulation of the distal part of the hypogastric nerve still elicited junction potentials in the smooth muscle cells of the guinea pig vas deferens although they were smaller and slower than normal. Their results might be due to fibers having a synaptic connection below the section and located in the vicinity of the stimulating electrodes. BURNSTOCK and HOLMAN further found that the rate of discharge of small spontaneous potentials was markedly slower in denervated preparations, which they explained as an effect of degeneration of sympathetic fibers. These findings might seem contradictory to the present results. It is possible, however, that the spontaneous discharge in non-denervated specimens is partly due to impulses from the cut nerves and the synaptic regions and that discharge of this type is abolished by denervation.

The location and type of these postulated synapses can at present only be a matter of speculation: they might be true ganglionic synapses located in the tissues adjacent to the vas deferens and the seminal vesicle or within them, or they might be synapses between neurons and chromaffin cells. Histochemical studies indicate an abundance of adrenergic nerve terminals in the vas deferens (Falck, personal communication). Thus it is not necessary to postulate that chromaffin cells are a source of the large amounts of noradrenaline remaining after section of the hypogastric nerves.

The present findings that high doses of reserpine releases noradrenaline from the ductus deferens and the seminal vesicle is in accordance with results obtained from other organs (BERTLER, CARLSSON and ROSENGREN 1956, MUSCHOLL and VOGT 1958, BURN and RAND 1958). The complete depletion of the noradrenaline is more indicative of a storage in adrenergic nerves than in chromaffin cells.

This investigation was supported by grants from Karolinska Institutet, Wallenberg Foundation and Stiftelsen Lars Hiertas Minne. The technical assistance of Mr. John Johnson is gratefully acknowledged. Reserpine (Serpasil) was kindly put at my disposal by CIBA A B, Vällingby.

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N O To be publ

Acta physiol scand 1962 56 381—384

From the Institute of Physiology University of Helsinki Finland

Erythropoietic Activity of Saline Washings of Blood Cells Subjected to Low Atmospheric Pressure in vitro

II Effect of partial hemolysis of the blood

By

YRSA v HELLENS EVA HIRSJARVI and RITA NIKIFOROW

Received 14 June 1962

Abstract

HELLENS Y v E HIRSJARVI and R NIKIFOROW *Erythropoietic activity of saline washings of blood cells subjected to low atmospheric pressure in vitro II Effect of partial hemolysis of the blood* Acta physiol scand 1962 *56* 381—384 — In order to test the effect of initial hemolysis on the formation of erythropoietins in vitro saline suspensions of partially hemolysed and non hemolysed washed blood cells were subjected to low atmospheric pressure and the erythropoietic activity tested by injecting samples of the saline into test rabbits The recipient animals in the non hemolysed group reacted to the injection with an increase in the erythrocyte and reticulocyte count while in the hemolysed group the injection was ineffective save for a transient reticulocytosis It is concluded that the active agent or its precursor is lost from the blood during initial hemolysis

The results of a previous study suggest that normal blood cells when subjected to low atmospheric pressure in saline suspensions lend an erythropoietic activity to the saline (HIRSJARVI 1958) It was however noted that if the blood samples were even slightly hemolysed before preparing the suspensions no activity could be detected after the low pressure exposure This encouraged us to carry out some further experiments on the role of hemolysis in the formation of erythropoietins in vitro

Table I Effect of injections of saline separated from low pressure exposed saline suspensions of non hemolysed blood cells on recipients' red cells and reticulocytes

Recipient no	Erythrocytes mill/µl				Reticulocyte %			
	Initial count	Deviation from the initial count			Initial count	Deviation from the initial count		
		2nd day	3rd day	4th–5th day		2nd day	3rd day	4th 5th day
1	44	+ 03	+ 03	+ 06	27	— 01	+ 07	—
2	47	+ 06	+ 09	—	17	+ 05	+ 30	— 01
3	53	+ 01	00	+ 02	19	+ 05	+ 04	+ 07
4	52	+ 03	+ 05	+ 04	27	+ 03	+ 19	+ 01
5	47	00	+ 02	+ 01	14	+ 25	+ 22	+ 36
6	40	+ 07	+ 06	+ 08	20	+ 07	+ 10	+ 16
7	46	+ 07	+ 01	+ 04	32	+ 06	+ 10	—
8	56	— 02	+ 02	00	18	+ 07	+ 06	+ 13
9	58	— 04	— 03	00	31	— 03	—	— 06
10	48	+ 01	—	+ 01	22	+ 02	—	+ 2
11	51	+ 02	+ 02	— 01	36	— 10	—	— 16
12	55	— 03	— 05	— 04	20	+ 76	+ 14	—
13	45	+ 05	00	+ 09	20	+ 09	+ 06	+ 70
14	50	+ 04	— 01	—	21	+ 08	+ 03	—
Mean	494 ± 014	+ 021 ± 009	+ 013 ± 011	+ 020 ± 009	223 ± 017	+ 063 ± 021	+ 121 ± 037	+ 097 ± 055

Material and Methods

As test animals healthy rabbits of both sexes were used Blood was drawn from the donor animals by heart punctures or in a few case from the marginal ear vein 30 ml were taken from each animal The blood samples of 3—4 animals were pooled plasma discarded and the cells divided into two parts One part was washed with 09 % saline non hemolysed group and suspended into a volume of saline equal the initial plasma volume The second portion of cells was washed with 040—045 % saline so that slight but not complete hemolysis was obtained hemolysed group The washings were discarded and the cells suspended in 09 % saline The non hemolysed and hemolysed cell saline suspensions were then subjected to an atmospheric pressure of 200—100 mm Hg for 1 1/2—2 hours at room temperature After the exposure the saline was separated and without delay injected intraperitoneally into untreated recipient rabbits 10—15 ml in each animal None of the injected samples showed visible hemolysis

The recipients red blood count (erythrocytes hemoglobin and reticulocyte percentage) were followed before the injection until found fairly stable, and determined on the first 4—5 days after the injection

Results

Ten rabbits received injections of low pressure saline prepared from partially hemolysed blood cells hemolysed group , while low pressure saline from untreated blood was injected into 14 animals non hemolysed group

Table II Effect of injections of saline separated from low pr ssure exposed saline susp nsions of blood cells subjected to previous partial hemolys.s on recipients red cells and reticulocytes

Re cipient no	Erythrocytes mill /μl				Reticulocy e			
	Initial count	Deviation from the init al count			Initial count	Deviation from the initial count		
		2nd day	3rd day	4th-5th day		2nd day	3rd day	4th-5th day
1	47	+ 01	+ 04	+ 01	26	+ 0.6	+ 05	—
2	53	— 01	— 02	+ 07	26	+ 20	— 03	+ 04
3	49	— 01	+ 02	— 02	28	+ 07	—	+ 01
4	56	— 05	— 08	— 07	—	—	—	—
5	51	— 02	— 03	+ 02	—	—	—	—
6	45	00	— 04	—	36	+ 04	— 06	—
7	48	+ 01	+ 02	+ 01	27	+ 07	— 04	—
8	51	— 03	+ 03	— 02	13	+ 09	+ 10	+ 04
9	50	+ 01	—	+ 01	14	+ 17	+ 05	— 03
10	44	00	00	00	37	— 06	— 03	+ 03
Mean	494 ± 011	—009 ± 006	—007 ± 013	+001 ± 010	259 ± 02?	+080 ± 025	+006 ± 025	+014 ± 068

A definite difference is seen between the reactions in the two groups In the *non hemolysed group* (Table I) most recipients reacted with a moderate or even marked increase in the number of erythrocytes which lasted for 4—5 days The hemoglobin values mostly varied with the erythrocytes though the changes were less marked consequently the mean corpuscular hemoglobin was usually slightly decreased Most recipients also showed a reticulocytosis though the day to-day variations of the reticulocyte percentage were rather large This type of reaction was previously observed in rabbits receiving plasma from anoxic donors or from blood exposed to low pressure in vitro (BONSDORFF and JALAVISTO 1948 HIRSJARVI 1953)

In the *hemolysed group* (Table II) the injection was followed by a slight decrease of the erythrocytes and hemoglobin As regards the reticulocytes there was in most cases an increase on the second day after the injection, but the values were levelled again on the third day A very similar reaction is seen in rabbits after injection of saline or plasma from untreated homologous blood (BONSDORFF and JALAVISTO 1948)

Comparison of the *erythrocyte values* in the two groups reveals an almost significant difference between both groups on the second day (Table I and II bottom line 0 05 > P > 0 02) whereas the difference on later days is insignificant If in both groups the erythrocyte deviations on 2—5 days are combined a statistically confirmed difference is obtained the mean value in the non hemolysed group is + 0 179 ± 0 055 and in the "hemolysed group — 0 050 ± 0 047 (P < 0 01)

Statistical comparison of the *reticulocyte values* (Table I and II, bottom line) gives a significant difference between both groups on the third day ($0.01 < P < 0.02$). If all the deviations after injection in each group are combined the difference borders on statistical probability mean value in the "non hemolysed group" is $+0.88 \pm 0.19$, and in the "hemolysed group" $+0.37 \pm 0.15$ ($0.05 < P < 0.1$)

These results suggest that saline suspensions of non hemolysed blood cells when subjected to low atmospheric pressure in vitro gain erythropoietic activity, whereas, if the blood is partially hemolysed before the preparation of the suspensions, no activity is gained during the low pressure exposure

Discussion

According to the data presented it seems evident that the substance responsible for the erythropoietic activity is derived from the blood cells most likely from erythrocytes, during the low pressure exposure. It also seems that the agent is lost from the cells in initial hemolysis. This conception is supported also by the observation that the discoloured saline washings in the "hemolysed group" in some cases showed erythropoietic activity after low pressure exposure. As an explanation for this it might be suggested that the precursor of the active substance is present in the young possibly more fragile erythrocytes but not in equal amounts in the older cells. This is in accord with the observation that blood from donors with increased erythropoiesis and presumably younger red cell population, gains a more potent erythropoietic activity during low pressure exposure than blood from donors with normal erythropoiesis (HIRSJÄRVI 1953). On the other hand, it is also conceivable that in the prehemolytic stage some change in the erythrocyte membrane takes place, which allows the active agent or its precursor to escape even from the non hemolysed cells so that these cannot, during the subsequent low pressure exposure, lend activity to the saline

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A Corneal Nipple Pattern in Insect Compound Eyes

by

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In electron microscope studies we have found that the corneal surface of the facets of certain insect compound eyes contain congruent cone shaped protuberances (Fig 1) These nipples are arranged in a more or less perfect hexagonal array that completely covers the corneal surface They are absent in extracorneal chitin Both the altitude of the nipples and their center to-center distance are approximately 200 mμ

This nipple pattern has been found on the corneas of compound eyes from insects belonging to Lepidoptera *i e* butterflies and moths *(Prodenia Cerapteryx Vanessa)* Neuroptera *i e* net flies *(Myrmeleon)* and Trichoptera *i e*, caddis flies *(Phryganea)* The pattern is absent in corneas of compound eyes of specimens belonging to Hymenoptera *i e* bees *etc (Apis Bombus)* Coleoptera, *i e* beetles *(Coccinella)* and Odonata *i e* dragon flies *(Sympetron)* Furthermore where the pattern is absent the front surfaces of these corneas appear smooth under the electron microscope Further investigations of the distribution and functional significance of the nipple pattern are in progress

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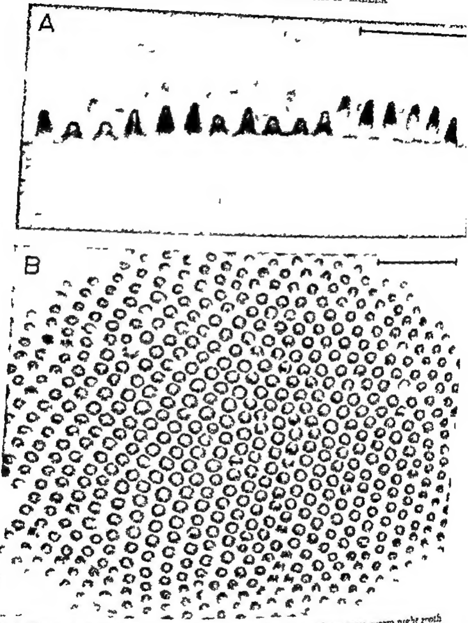

Fig 1 Electron micrographs of corneal nipple pattern in the southern army worm night moth (*Prodenia eridiana*) A Section normal to corneal facet surface B Section tangent to corneal facet and slightly above the surface Scale 1 μ in both micrographs